NOV 2 1922 Medical Lib.

VOL. III. No. 5] [OCTOBER, 1922.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

Editors:

C. L. EVANS
P. FILDES
W. E. GYE
E. H. KETTLE
J. McINTOSH
H. MACLEAN
J. A. MURRAY
J. B. ORR.
W. J. TULLOCH
C. M. WILSON

With the Support of:

F. W. ANDREWES (London)
W. M. BAYLISS (London)
W. BULLOCH (London)
S. L. CUMMINS (Cardiff)
H. H. DALE (London)
A. M. DRENNAN (Otago, N.Z.)
G. DREYER (Oxford)
J. W. H. EYRE (London)
LEONARD FINDLAY (Glasgow)
DUNCAN GRAHAM (Toronto)
R. T. HEWLETT (London)
F. G. HOPKINS (Cambridge)
W. B. LEISHMAN (London)
T. J. MACKIE (Cape Town)
H. OERTEL (Montreal)
LEONARD ROGERS (London)
C. S. SHERRINGTON (Oxford)
A. E. WRIGHT (London)

LONDON: H. K. LEWIS & CO. LTD., 136, GOWER STREET, W.C.1.
EDITORIAL AND PUBLISHING OFFICE: 28, GOWER PLACE, W.C.1.
CANADA: THE MACMILLAN COMPANY OF CANADA, LTD., BOND STREET, TORONTO.

Annual Subscription, post free, Two Pounds net.
Single Numbers, Seven and Sixpence net.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

EDITORIAL NOTICE TO CONTRIBUTORS.

Scope of Papers.—This Journal has been founded for the publication of original communications describing the technique and results of experimental researches into the causation, diagnosis and cure of disease in man. Papers upon bacteriological, biochemical, physiological, serological and other subjects are therefore acceptable when bearing directly upon these objects.

Forwarding Papers.—Papers should be addressed to—

THE EDITORS,
British Journal of Experimental Pathology,
28, GOWER PLACE,
LONDON, W.C. 1,

or to any of the Editors personally.

Date of Publication.—The Journal is published bi-monthly, in February, April, June, August, October, and December.

Priority of Publication.—Papers accepted for publication will be printed as far as possible in the order of their receipt by the Editors, but those sent from overseas will receive priority in publication according to the date of posting instead of the date of receipt.

Conditions of Publication.—Contributors should prepare their manuscripts with the greatest attention to detail. The fact of submitting a paper for publication will be taken by the Editors as an admission by the Author of his liability to pay for all unreasonable alterations made by him in proofs, and for mistakes due to lack of clearness in the preparation of the manuscript.

References.—Authors are requested to prepare their references in strict accordance with the method adopted in current issues of this Journal.

Proofs.—To avoid delay, proofs will not be submitted to Authors overseas unless they express a desire to see them, or unless the Editors think it necessary. Overseas Authors are therefore particularly reminded of the importance of preparing their manuscripts in such a way that error need not arise.

Reprints.—25 reprints will be supplied free to authors. Additional copies must be ordered from the Publishers *before the issue is printed,* and will be charged for according to the following scale:

Number of pages.	*Number of Additional Reprints.* 25	50	75	100	150	200
	£ s. d.	£ s. d.	£ s. d.	£ s. d.	£ s. d.	£ s. d.
1–4	0 16 6	0 19 6	1 2 6	1 5 6	1 9 6	1 13 6
5–8	1 5 0	1 8 0	1 11 0	1 14 0	1 19 6	2 5 0
9–12	1 16 0	1 19 0	2 2 0	2 5 0	2 12 6	3 0 0
13–16	2 5 0	2 8 0	2 11 0	2 14 0	3 6 0	3 15 0

Authors working in countries where the rates of exchange are unfavourable to them will be charged for reprints according to pre-war rates (25 francs = 20 shillings).

IMPORTANT NOTICE.

CONTRIBUTORS are asked to observe that papers exceeding 4500 words in length can only be published when space is available and not in the order of receipt by the Editors. This limitation has been found necessary to avoid undue delay in the publication of results.

The Editors urge all contributors, whatever the length of their communications, to reduce to a minimum reviews of previous work and theoretical discussions of their results.

THE BRITISH JOURNAL
OF
EXPERIMENTAL PATHOLOGY

THE RELATIVE VOLUME OF CORPUSCLES AND PLASMA, AND THE RELATION OF THIS TO HÆMOGLOBIN PERCENTAGE AND THE NUMBER OF RED BLOOD-CORPUSCLES.

J. M. H. CAMPBELL,
Hilda and Ronald Poulton Research Fellow at Guy's Hospital, London.

Received for publication May 16th, 1922.

WHILE using the hæmatocrit for some routine determinations of the relative volume of red cells in whole blood, it was obvious that this ratio depended less on the number of red cells present than on the hæmoglobin percentage.

The hæmatocrit was first used for this purpose by Heden (1891), and, more extensively, by Capps (1903), who introduced the term "volume index." This is obtained by dividing the relative volume of the corpuscles by the number of red cells (both being expressed as percentages of the normal), and gives a measure of the relative size of the red cell. Gram (1921) has recently published a somewhat similar investigation, 5 c.c. of blood being rotated in the centrifuge for ninety minutes. Biernacki (1894) allowed sedimentation of the corpuscles for twenty-four hours after adding potassium oxalate. Larrabee (1911), using this method, confirmed many of the results obtained by Capps.

METHODS.

Venous blood was used for all experiments unless otherwise stated. Solid potassium oxalate sufficient to give a 0·5 per cent. solution was added.

In some experiments where blood was obtained in the usual way by pricking the finger and adding minimal quantities of potassium oxalate, it was much more difficult to get constant results, owing to the impossibility of getting any constant percentage of potassium oxalate (see appendix).

The hæmoglobin percentage was determined generally in duplicate by a specially standardised Haldane-Gowers hæmoglobinometer.

The red blood-corpuscles were counted with a Bürker-Zeiss hæmocytometer and at least two thousand were counted in each case.

The following method was used to determine the relative volume of corpuscles. Glass tubing was drawn out to capillary bore. Any part with obvious changes in diameter was thrown away, and the remaining length was cut up into capillary tubes of the required length. The blood was well mixed,

and six tubes were dipped into the blood till it had risen to near the top. The upper end was then sealed in a flame, care being taken not to burn the part containing blood.

The same centrifuge was always used at the same speed; the six tubes used in each determination were rotated for fifteen minutes, which was found sufficient to give approximately constant results.

The heights of the corpuscles and of the whole blood were then measured and the former expressed as a percentage of the latter. By using six tubes and taking the mean errors were minimised. The following figures illustrate this point, and give details of some other experiments which were done to test the validity of the method.

In one experiment after centrifuging for fifteen minutes a value of 47 per cent. was obtained. In a second experiment six tubes filled with the same blood were spun for seven and a half minutes, when a rather higher result was obtained—52·7 per cent. Obviously, sedimentation was not complete. The same tubes were then rotated for a second seven and a half minutes, when the result obtained was about the same as in the first experiment, *i. e.* 47·9 per cent. In a third experiment after rotating another six tubes with the same blood for fifteen minutes the result was 47·5 per cent. The same tubes were then spun for another ten minutes, when the value was only reduced to 46·9 per cent. In these three separate determinations on the same blood the values found were 47·0, 47·5, and 47·9.

In another experiment blood was taken from a subject on successive days with the following results: 34·5 per cent. and 36·3 per cent. A much greater difference than this was not found in any cases where separate determinations were made.

As regards the variations in individual tubes four consecutive experiments will give a good idea of this:

i. 17·6, 16·2, 18·0, 21·5, 16·2, 17·6, 17·0, 18·8 = av. 18·0.
ii. 70·9, 66·3, 68·2, 64·9, 67·4, 66·3, 66·1, 68·1 = av. 66·9.
iii. 46·2, 43·6, 41·9, 35·5, 41·1, 38·9, 35·2, 36·7 = av. 40·0.
iv. 28·2, 29·8, 31·1, 29·8, 26·7, 29·7, 32·9, = av. 29·7.

Obviously single determinations, which have usually been used by previous workers, could only give a rough indication, but the use of six tubes gives a more reliable figure. The reading obtained is accurate within 5 per cent.—an error greater than that of the Haldane-Gowers hæmoglobinometer and less than that of an ordinary red cell count.

Other experiments were done with capillary tubes which were obviously not of equal bore, in three tubes the corpuscles being at the broader end and in three at the narrower end. The results were as follows, in three separate experiments, six tubes being used each time: At the thick end, 35·1, 19·4, and 32·1; at the thin end, 39·1, 23·3, and 40·2. In the last case the different bore was very obvious, and in the two other cases the difference would have been recognised at once by inspection, and the tubes would not have been used in the ordinary routine.

One criticism which was urged against the use of this method was that the cells were subjected to too much pressure by rotating in the centrifuge. It was thought that no estimate of their true size could be obtained, and all that would be left would be the hæmoglobin and stroma, all fluid being forced out. To investigate this point the following experiment was performed. Some blood was centrifuged, and equal volumes of corpuscles were then mixed with equal volumes of 0·6 per cent. and 0·9 per cent. NaCl solutions. The former swelled up but hæmolysis did not take place. After standing for half an hour to reach equilibrium the mixtures were stirred up and sets of capillary tubes were filled with each mixture. These were centrifuged and measured in the usual way. In three experiments the following results were obtained with blood from different subjects; after mixing with 0·6 saline, 25·1 per cent., 15·0 per cent., and 37·2 per cent.; after mixing with 0·9 saline, 23·1 per cent., 12·6 per cent., and 31·6 per cent. In each case the blood with larger corpuscles gave a larger value for the relative volume of corpuscles, and in the third case, at any rate, the result was outside the limits of experimental error.

If the method is valid as a means of measuring the relative sizes of corpuscles which only differ as the result of osmotic change, it is certainly valid to measure variations in size, which depend on a more fundamental change in the nature of the corpuscles, as in pernicious and secondary anæmias. In any case the conditions observed in each experiment were strictly

the same, so that the compression during rotation would tend to produce a similar degree of change in each experiment. Even if the absolute size of the corpuscle is somewhat modified a comparison between the size in various conditions may still be made.

RESULTS.

Sixteen subjects with no anæmia were used as normal controls. The results are given in full in the appendix. The number of red cells, the hæmoglobin and the corpuscular volume are all expressed as percentages of the normal. In column 7 is given the "hæmoglobin index." It is obtained by dividing the hæmoglobin by the corpuscular volume and measures the concentration of hæmoglobin in the corpuscle.

Secondary anæmia.—In eleven cases of anæmia, mostly due to loss of blood, the red cells averaged 62 per cent., hæmoglobin 34 per cent. (12–56), and corpuscular volume 39·5 per cent. The colour index averaged 0·56 (0·38–0·83), and the volume index 0·66. The hæmoglobin index was 0·86—much nearer the normal than any of the other results. There is a reduction of about 30 per cent. in the average size of the red cells, but the concentration of hæmoglobin in the corpuscle is reduced only 10 per cent.

Chlorosis.—In eight cases of chlorosis the red cells averaged 70 per cent., the hæmoglobin 41 per cent. (19–68), and the corpuscular volume 51 per cent. The colour index averaged 0·56 (0·42–0·78), and the volume index 0·70. The hæmoglobin index was 0·80, relatively constant and only slightly below the corresponding figure for the secondary anæmias. The reduction in the size of the red cells is about the same.

Pernicious anæmia.—In seven cases of pernicious anæmia the red cells averaged 29 per cent.; hæmoglobin 34 per cent. (16–65), and corpuscular volume 41 per cent. Colour index averaged 1·24 and volume index 1·39. The hæmoglobin index averaged 1·00 and varied between 0·82 and 1·35. Thus the average size of the corpuscles was increased by 30 per cent. above normal, while the concentration of the hæmoglobin in each corpuscle was slightly (4 per cent.) above normal. In severe polycythæmia due to congenital heart disease, where the number of red cells is about doubled, there was no change in the colour index, in the size of the corpuscles, or in the concentration of hæmoglobin in the corpuscle. The same was found to be true in cases of acholuric jaundice. Taking these groups as a whole the most striking point is the relative constancy of the "hæmoglobin index." While the red count varies between 29 and 163 per cent. and the hæmoglobin between 34 and 140 per cent., the colour index varies between 0·56 and 1·20 and the volume index between 0·66 and 1·30, but the hæmoglobin index only varies between 0·80 and 0·96.

With changes of over 100 per cent. in the number of red cells and the hæmoglobin percentage, the colour index and size of the red cells vary over 66 per cent., but the concentration of hæmoglobin in each corpuscle only varies 16 per cent. It is almost, but not quite, true to say that the size of the corpuscle depends entirely on its hæmoglobin content.

If individual cases are considered instead of groups, the results are almost equally striking. The red cell count varies from 12 per cent. to 186 per cent., the hæmoglobin from 12 to 155 per cent., and the corpuscular volume from 15 to 176—very similar ranges of variation. The changes in the colour index

and the volume index are much less, but very considerable. The former lies between 0·38 and 1·5 and the latter between 0·46 and 1·80, or, if one case is excluded, 1·50.

But the hæmoglobin index which measures the concentration of hæmoglobin in the corpuscle only varies between 0·65 and 1·35. If four extreme cases are excluded—and two of these results are not considered equally reliable, as they were two of the six results obtained on capillary blood—the hæmoglobin index only varies between 0·75 and 1·15.

It is clear that in various pathological conditions the number of red cells is the most important factor in maintaining the hæmoglobin percentage; that the size of the red cells is the second most important factor; and that changes in the concentration of hæmoglobin in the corpuscle only occur to a slight extent and are relatively unimportant. The colour index is more nearly than had been supposed a measure of the average size of the red cells.

It is often stated that chlorosis is characterised by the particularly low colour index and by the pale corpuscles. The colour index in these groups is the same, and if each determination is plotted on a graph with colour index as ordinates and hæmoglobin percentage as abscissæ, there is in general a decrease of colour index with a decrease of hæmoglobin percentage, and the points representing chlorosis are not obviously lower than the points representing the secondary anæmias.

DISCUSSION.

Corpuscular volume.—In normal cases this varied between 32 and 45·6, but only in two cases was it below 36 and only in two cases was it above 42. It averaged 40 per cent. Capps (1903) found 48 per cent. and Larrabee (1911) 50 per cent., but the latter did not use a centrifuge, and the former did not add any reagent to prevent clotting.

Gram (1921) used a method more closely resembling this, and found that it varied closely with the hæmoglobin, the average result in normal cases being hæmoglobin 90 per cent. and corpuscular volume 42 per cent.

Volume of the individual red corpuscle.—In some ways this method is more accurate than the measurement of the diameter of the cells because it is the true volume which is measured, and such large numbers of cells are used and an average taken. A comparison between the two methods has been made by several observers.

Fischer (1915) found that 6·2 μ (diameter) corresponded to a corpuscular volume of 33·8; 6·4 μ to 35·0; and 6·7 μ to 39·0. Price-Jones (1920), using solutions of sodium bicarbonate, lactic acid and normal saline, found that a change in reaction produced changes in the size of the corpuscle. Averaging three of his separate experiments he found that 6·2 μ corresponded to 92 per cent.; 6·3 μ to 100 per cent.; and 6·5 μ to 113 per cent. Capps (1903) measured the mean diameter in a large number of pathological conditions, and found that it was 8·0 μ in cases of pernicious anæmia, 7·65 μ in normals, and varied between 7·1 μ and 7·5 μ in different groups of secondary anæmias and chlorosis. In pernicious anæmia Price-Jones found a mean diameter of 8·2 μ in dry films.

The diameter of the red cells in these cases was not measured, but suppos-

ing the increase took place equally in all diameters, an increase of 10 per cent. in volume is produced by an increase of just over 3 per cent. in diameter. Supposing there is not much change in the thickness of the corpuscle, an increase of 10 per cent. in the volume of the corpuscle would be produced by rather less than 5 per cent change in diameter, *e. g.*:

$$\text{If } v = kr^3 \text{ and } R = 1{\cdot}033\ r$$
$$V = kR^3 = k\ 1{\cdot}10r^3 = 1{\cdot}10\ v,$$
$$\text{and if } v = Kr^2 \text{ and } R = 1{\cdot}05\ r$$
$$V = KR^2 = K\ 1{\cdot}10\ r^2 = 1{\cdot}10\ v,$$

where v, r, V and R are the volume and radius of the ordinary and of the larger corpuscle respectively. The true value must lie between these limits.

In this series the volume index was increased 30 per cent. in anæmias of high colour index and diminished 30 per cent. in anæmias of low colour index. This means that the mean diameter varied in these two groups by 9–15 per cent. from the normal. Taking $7{\cdot}7\ \mu$ for the normal the mean diameter in these cases of high colour index would be about $8{\cdot}4\ \mu$, and in the cases of lower colour index about $7{\cdot}0\ \mu$.

All these methods agree in demonstrating the increased size of the average cell in pernicious anæmia in spite of the presence of microcytes. In chlorosis and secondary anæmias the average size of the red cells is reduced.

CONCLUSIONS.

(1) A method is described for estimating accurately the volume of red corpuscles in whole blood. In normals this was found to be 40 per cent.

(2) The corpuscular volume varies almost directly with the hæmoglobin percentage. There is little change in the concentration of hæmoglobin in the corpuscle in various pathological conditions.

(3) The term "volume index," which is the ratio of the corpuscular volume to the number of red cells, gives a measure of the relative size of the red cells. The size depends almost entirely on the hæmoglobin content. From (2) and (3) it follows that the colour index is a rough guide to the size of the average red cell.

(4) In pernicious anæmia the average cubic contents of each red cell is increased by about 30 per cent. In secondary anæmias and chlorosis it is diminished by about 30 per cent. In polycythæmia it is unchanged.

(5) The diameter of the corpuscles calculated from the volume agrees fairly closely with the direct measurements made by other observers.

(6) From many points of view the blood changes in chlorosis are exactly the same as in the secondary anæmias, and do not resemble in any way the changes in the other so-called primary anæmia.

Many of these conclusions follow from the very extensive investigations carried out by Capps, but his paper does not seem to have received the attention it deserves.

Gram was the first to point out clearly that the close parallelism between the colour index and the volume index indicated a constant concentration of hæmoglobin in the corpuscle: "Il en résulte que les écarts pathologiques de l'indice colorimetrique sont dus essentiellement à des variations du volume moyen des globules."

APPENDIX.

1. —	2. Red blood-corpuscles, percentage of normal.	3. Hæmoglobin, percentage of normal.	4. Corpuscular volumes, Percentage of normal.	5. Colour index.	6. Volume index.	7. Hæmo-globin index.
1. Healthy student	114	86	104	0·75	0·91	0·83
2. „	118	99	107	0·84	0·90	0·93
3. „	118	86	105	0·73	0·89	0·82
4. „	106	84	82	0·75	0·73	1·02
5. „	108	96	92	0·83	0·79	1·04
6. „	98	96	92	0·98	0·94	1·04
7. „	110	87	97	0·79	0·88	0·90
8. Cerebral tumour	102	88	90	0·86	0·88	0·98
9. Carcinoma stomach	92	91	97	0·99	1·03	0·93
10. Cerebral hæmorrhage	102	108	101	1·06	1·00	1·06
11. Recovered chlorosis	100	85	97	0·81	0·94	0·81
12. Emphysema	96	95	90	0·99	0·94	1·06
13. Heart failure	84	86	90	1·02	1·07	0·95
14. Hodgkins' disease	102	90	101	0·88	0·98	0·90
15. Emphysema	90	82	80	0·90	0·89	1·01
16. Mitral stenosis	112	108	114	0·97	1·02	0·95
Average (1–16): 16 normals	103	92	100	0·89	0·97	0·96
17. Gallstones	86	70	77	0·81	0·90	0·91
18. Heart failure	66	70	70	1·06	1·06	1·00
19. Menorrhagia	84	42	50	0·50	0·60	0·84
20. Gastric ulcer	98	45	52	0·46	0·54	0·86
21. Sarcoma kidney	68	29	32	0·43	0·53	0·90
22. „	34	13	15	0·38	0·46	0·87
*23. Pyorrhœa	80	56	57	0·70	0·71	0·98
24. Gastric ulcer	46	33	34	0·72	0·74	0·97
*25. „	38	31	39	0·83	(1·04)	0·79
26. „	76	44	45	0·58	0·59	0·98
27. Chronic nephritis	64	39	47	0·61	0·71	0·82
28. Ulcerative colitis	70	32	42	0·45	0·60	0·75
*29. Carcinoma	30	12	18	0·40	0·62	(0·65)
Average (19–29): Secondary anæmia	62	34	39·5	0·56	0·66	0·86
*30. Chlorosis	98	50	62	0·50	0·62	0·80
31. „	90	51	59	0·56	0·65	0·86
*32. „	92	68	86	0·75	(0·92)	0·79
33. „	45	19	29	0·42	0·64	(0·65)
34. „	52	30	35	0·58	0·68	0·84
35. „	60	27	33	0·45	0·56	0·81
36. „	48	25	31	0·50	0·65	0·81
37. „	74	58	70	0·78	(0·94)	0·83
Average (30–37): Chlorosis	70	41	51	0·56	0·70	0·80
38. Infective endocarditis	34	34	36	1·00	1·06	0·94
39. Pernicious anæmia	32	35	35	1·12	1·10	1·00
40. „	18	27	27	1·50	1·52	1·00
41. „	23	25	27	1·08	1·20	0·91
42. „	12	16	18	1·34	1·50	0·94
43. „	20	27	31	1·35	1·55	1·15
44. „	48	65	87	1·36	1·80	1·35
45. Ditto (after transfusion)	50	48	59	0·96	1·18	0·82
Average (39–45): Pernicious anæmia	29	34	41	1·24	1·39	1·00

APPENDIX—(*continued*).

1.	2. Red blood-corpuscles, percentage of normal.	3. Hæmoglobin percentage of normal.	4. Corpuscular volumes, percentage of normal.	5. Colour index.	6. Volume index.	7. Hæmo-globin index.
46. Congenital heart disease .	164	152	171	0·93	1·04	0·89
47. „ „ .	186	145	167	0·78	0·90	0·87
48. „ „ .	132	106	118	0·80	0·89	0·90
49. „ „ .	170	155	176	0·91	1·02	0·88
Average (46–49): Congenital heart disease	163	140	158	0·86	0·97	0·89
*50. Acholuric jaundice . .	36	38	30	1·06	0·85	(1·24)
51. „ . .	54	50	56	0·93	1·04	0·90
52. „ . .	70	62	74	0·90	0·90	0·84
Average (50–52): Acholuric jaundice .	53	50	53	0·97	0·93	0·97
Present Series.						
Normals	103	92	100	0·89	0·97	0·96
Secondary anæmia . . .	62	34	39·5	0·56	0·66	0·86
Chlorosis	70	41	51	0·56	0·70	0·80
Pernicious anæmia . . .	29	34	41	1·24	1·39	1·00
Capps (1903).†						
Normals	96	86	95	0·90	0·99	0·91
Secondary anæmia . . .	60	43	50	0·75	0·84	0·82
Slight anæmia	87	70	82	0·85	0·95	0·89
Severe anæmia	44	33	42	0·80	0·95	0·84
Very acute anæmia . . .	50	28	36	0·60	0·72	0·83
Chlorosis	81	49	66	0·66	0·82	0·80
„ slight	89	63	82	0·78	0·92	0·85
„ severe	74	36	53	0·54	0·72	0·75
Pernicious anæmia . . .	29	32	40	1·27	1·37	0·92
Gram (1921).						
Normals	—	—	—	0·90	1·0	0·90
Anæmia of low colour index .	—	—	—	0·60	0·8	0·75
Pernicious anæmia . . .	—	—	—	1·45	1·5	0·97

NOTES TO APPENDIX.

* The six results marked thus were obtained with capillary instead of with venous blood. Six figures have been included in brackets where the result seemed to be well outside the range of variation for that group. In obtaining the average figure these results have all been included. They have not been included in the range of variation which is discussed in the text of the paper, especially as four of these six results were obtained on capillary blood.

† The colour index has not been calculated in exactly the same way, so that these results are not strictly comparable.

I wish to thank Mr. Reeves and Mr. Cohen for the results on the seven students, Dr. Poulton for many suggestions and for the loan of apparatus

purchased with a grant from the British Medical Association, and Dr. Laidlaw for the use of his laboratory and for much assistance and useful criticism.

REFERENCES.

BIERNACKI, E.—(1894) *Ztschr. f. klin. Med.*, **24**, 460.
CAPPS, J. A.—(1903) *J. Med. Res.*, **10**, 367.
FISCHER, M.—(1915) 'Œdema and Nephritis,' London (Chapman & Hall).
GRAM, H. C.—(1921) *Compt. rend. Soc. de Biol.*, **8**, 151.
HEDEN, S. G.—(1891) *Skand. Arch. f. Path.*, **2**, 135, 360.
LARRABEE, R. C.—(1911) *J. Med. Res.*, **24**, 15.
PRICE-JONES, C.—(1920) *J. Path. & Bact.*, **23**, 371.—(1921) *Ibid.*, **24**, 326.

ON THE ANTITRYPTIC ACTION OF THE BLOOD.

E. BEATON.

From the Public Health Laboratories, Cairo.

Forwarded for publication May 11th, 1922.

THE capacity of the blood-serum to inhibit the action of trypsin presents a problem requiring further investigation. The antitryptic power may be measured with a considerable degree of exactitude, and the interest of the function is increased by the fact that, while confined within certain limits in normal individuals, it is found to be enhanced beyond those limits in certain diseases. Since its first observation by Hahn in 1897, the inhibitory power has excited a good deal of attention from physiologists and pathologists, and several hypotheses have been put forward to explain its nature. It was at first assumed to be an evident immunity reaction, but later doubt was cast on this view, and various conceptions of a less complex character were adduced to explain the effect. The view of antitryptic action which appears to prevail at present, which may be called the physiological view, since it embodies the explanation of the phenomenon presented by Bayliss (1919) and by O. Cohnheim (1912), is that the reduction of tryptic action produced by serum is due to a diversion of the enzyme from the added substrate by the proteins of the serum, these proteins being assumed to be themselves acted upon by trypsin only slowly and with difficulty, but to be able to adsorb the enzyme and so to diminish its effective concentration. The implication of this conception is that there is no justification for regarding the antitryptic factor in the serum as an anti-enzyme; that the phenomenon is sufficiently elucidated by the interpretation of the manner of the inhibition which the conception affords, and that the only problem remaining for consideration lies, not in the inhibitory effect itself, but in the resistance of the serum-proteins to the action of the enzyme. This being so, the question becomes a purely physiological one and loses much of its interest to the pathologist. It is, however, my purpose here to suggest that the subject is not one to be lightly

dismissed as having been adequately explained by the hypothesis of simple adsorption, but that we have under consideration a problem of no small complexity and importance, and one from the further examination of which we may reasonably hope to increase our knowledge of the metabolic processes in health and disease. The main impediment to the acceptance of the simple explanation lies in the occurrence of the pathological variations. It is a fact that in certain conditions of disease the inhibitory power may depart appreciably, and often considerably, from the normal level, returning gradually to this level when the exciting factor has been removed, and any explanation of antitryptic action must take cognisance of this liability of the function to variation and must be compatible with it.

It will be apparent that there are several directions in which the further investigation of the problem might be undertaken. Having regard, however, to the hypothesis under consideration, it has seemed expedient in the first place to examine the relation existing between the serum-proteins and the antitryptic power. This examination has been undertaken by several investigators with regard to one point, namely, the association of the normal inhibitory action with the albumin and globulin fractions of the protein; but it does not appear to have been extended to the abnormal increment of the antitryptic factor, which would not *à priori* necessarily be in this respect identical with the normal factor; nor does any inquiry appear to have been made as to whether there exists a quantitative relation between the serum-proteins and the antitryptic power. As these two points are also of importance in their bearing upon the nature of the inhibitory factor, the following observations are put forward as having been directed towards their elucidation.

METHOD.

A number of different procedures have been employed for the determination of the antitryptic power of the serum, all of which fall, however, into one of two categories, based on two different principles. In the methods of the first category, serum is added to an arbitrary excess of trypsin in the presence of a suitable substrate, and the amount of resultant digestion is determined in comparison with the amount produced by the same quantity of enzyme in the absence of the serum. Most of these methods require relatively large quantities of the reagents, including the serum, and although undoubtedly measuring the amount of inhibition under the conditions employed, are not necessarily to be regarded as affording an expression of the true quantitative relation between the enzyme and the inhibitory factor. The methods of the second category constitute practically a titration of the inhibitory factor against the trypsin. In these, graduated dilutions of the trypsin solution are mixed with uniform quantities of the serum under examination; a suitable substrate is then added, incubation maintained for a fixed period, and the result read by noting the presence or absence of an observable degree of digestion in the tubes. The requirement of the substrate is that it should afford readily observable evidence of digestion, and various substances have been used for this purpose; but the most generally useful I have found to be a 1 per cent. solution of caseinogen. This gives a heavy precipitate on acidification, but it

is readily converted by trypsin into a form in which the precipitate is no longer produced, and the solution after the addition of acid remains clear. If the serum under examination be diluted to 1 part in 10, and the graduated dilutions of trypsin employed be made to differ by one-tenth of their value, a very sharp end-point will be obtained, and the inhibitory power of different samples of serum may be readily compared and expressed in terms of the quantity of the given trypsin solution that has been neutralised by each. This method, which was originally devised by Fuld, and independently by Gross,* has been adversely criticised, but I have found it constant in its results and applicable to small quantities of serum. The measurements obtained have, of course, been only comparative, but have been adequate for the purpose of the investigation.

THE RELATION OF THE NORMAL ANTITRYPTIC FACTOR TO THE PROTEINS OF THE SERUM.

The investigations of Cathcart (1904), Doblin (1916), Kurt Meyer (1911) and Teale and Bach (1920) have shown that the greater part of the antitryptic power lies in the albumen-fraction of the serum-proteins, Cathcart concluding that the whole of the inhibitory factor is in this fraction and that the globulin is not antitryptic. My own experience has indicated that although the partition of the inhibitory effect between the two fractions is not uniform throughout the various animal species, the albumen is always more inhibitory than the globulin, and that in the case of human serum the association of the effect with the albumen-fraction is very pronounced. The difference between the two fractions in this respect is shown in Table I.

THE QUESTION OF WHETHER THE ASSOCIATION OF THE INHIBITORY FACTOR WITH THE ALBUMEN-FRACTION APPLIES TO THE PATHOLOGICAL INCREMENT OF ANTITRYPSIN.

The protein-fractions were prepared by precipitation with ammonium sulphate. After half-saturation with this salt the globulin was filtered off, redissolved, and again precipitated. The two filtrates from the globulin were completely saturated with the salt, and the albumen separated by filtration. Each precipitate was then dissolved in such a volume of saline that the solution corresponded to a definite dilution of the original serum, and the strength of the solutions will be expressed in this way. Thus, a 1 in 10 solution of albumen will denote the albumen of 1 c.c. of serum dissolved in such a volume of saline as to give a final volume of 10 c.c. The solutions necessarily contained a certain amount of ammonium sulphate, which was not removed, as similar solutions of the salt alone were found to have no antitryptic action.

It will be seen in Table I that although practically the whole of the antitryptic power was in the albumen, the power of the albumen was less than that of the whole serum. This I have found to be constant. The loss is not, however, due to the absence of the globulin, since, in the case of human serum,

* Described by von Bergmann and Meyer (1908). I have not adhered strictly to the original technique.

the inhibitory strength of the albumen is not increased by adding the globulin to it. The reconstructed serum thus formed has always inhibited the same amount of enzyme as the albumen alone. In the case, however, of those animals referred to above, in which the inhibitory power of the globulin is greater, the inhibitory power of the albumen is found to be increased by the addition of the globulin, but the mixture never attains the inhibitory power of the whole serum. The reduction is presumably due to an alteration in the albumen produced by the precipitation.

The question now under consideration is elucidated by Table I. It is seen there that the increment of the antitryptic factor in the abnormal sera B and D has the same relation to the protein-fractions as the normal factor in A and C. The "antitryptic indices" of the fractions are approximately the same as those of the whole sera, and the index B/A derived from the albumen is seen to be identical with that derived from the reconstructed sera made by mixing the two fractions. The slight discrepancy existing is probably due to experimental error, and is not altogether surprising, in view of the fact that a complicated quantitative procedure has been carried out on a small amount of material.

TABLE I.—*The Antitryptic Power of the Protein-Fractions of Sera having Different Inhibitory Capacities.*

	A.	B.	B/A.	C.	D.	D/C.
Serum 1 in 10 . . .	21	38	1·8	21	38	1·8
Albumen 1 in 10 . . .	17	29	1·7	19	32	1·7
Globulin 1 in 5 . . .	3	5	1·7	3	5	1·7
Albumen + globulin 1 in 10 .	17	29	1·7	—	—	—

A, B, C and D represent four samples of serum. The figures under A, B, C and D indicate the number of c.mm. of trypsin-solution just neutralised by 100 c.mm. of the solution of protein, 100 c.mm. of a 1 per cent. solution of caseinogen having been added as the substrate.

The figures do not suggest that the determination was always carried out to the nearest c.mm., but denote, in terms of whole numbers, the quantity of undiluted trypsin-solution in the logarithmic series of dilutions employed, these dilutions differing, as nearly as practicable, by one-tenth of their value.

The conclusion to be drawn is that, in human serum, the pathological increment is also contained almost entirely in the albumen-fraction of the serum protein, and that when two sera differ in their antitryptic power, the albumen-fractions differ in this respect to the same extent. The investigation of the quantitative relation between the serum-protein and the antitryptic power has, in consquence, involved a separation of the protein-fractions and a determination of the amount of each fraction in the various samples of serum examined.

THE QUESTION OF THE RELATION OF THE ANTITRYPTIC POWER TO THE QUANTITY OF ALBUMEN IN THE SERUM.

The relation of the antitryptic power to the quantity of albumen, and also to the quantity of globulin, is shown in Table II. In these observations the

determination of the protein was carried out by the Kjeldahl method, and herein an objection arose to the use of ammonium sulphate as the precipitant, since it was found very difficult to remove completely the salt from the filters. Hence, magnesium sulphate was used to separate the globulin, as, although it has been shown that the quantities of protein precipitated by saturation with magnesium sulphate and by half-saturation with ammonium sulphate are not necessarily identical, the agreement was thought to be sufficiently close for the purpose of the investigation. The globulin was, therefore, precipitated by magnesium sulphate and re-dissolved; and the separated fractions were coagulated by heat, filtered off, washed, and digested in sulphuric acid on the filter-papers. Only 1 c.c. of serum was used for this estimation, but the result was always controlled by the determination of the total coagulable protein in 0·5 c.c.

It will be seen from Table II that there is no proportional relation between the inhibitory power and the quantity, either of the total protein or of the albumen, but that the tendency is for sera with a high antitryptic index to have a low protein content and especially a low albumen content. The antitryptic power was, in these experiments, determined from the whole serum, but assuming the relative inhibitory capacities of the albumen-fractions to have been, as usual, the same as those of the whole sera, we see, on comparing the first sample with the last, that the albumen of the last had in unit mass nearly six times the inhibitory power possessed by the albumen of the first.

TABLE II.—*The Relation of the Antitryptic Power to the Quantity of Albumen and of Globulin in the Serum.*

Antitryptic index of serum.	Grm. in 100 c.c.		Per cent. of protein.		Total protein. (as control).
	Albumen.	Globulin.	Albumen.	Globulin.	
1·0	5·0	3·4	60	40	8·3
1·0	4·6	2·4	66	34	7·4
1·0	4·2	2·6	62	38	7·0
1·1	3·2	4·0	44	55	7·4
1·4	3·6	2·9	55	45	6·7
1·5	3·1	4·0	44	56	7·2
1·7	2·4	3·5	41	59	5·9
1·7	3·1	2·9	51	49	6·0
1·9	3·2	3·2	50	50	6·5
2·0	1·8	4·6	28	72	6·5

THE QUESTION OF THE RELATION BETWEEN THE ANTITRYPTIC POWER OF THE SEPARATED ALBUMEN AND THE CONCENTRATION OF THE ALBUMEN IN THE SERUM.

Here, in order to meet criticism on the ground that the relative inhibitory power of the different sera might not always be identical with that of their albumen-fractions, the albumen, as well as the globulin, was precipitated and redissolved, and the actual inhibitory power of the albumen was determined. It was, therefore, necessary to use ammonium sulphate as the precipitant, and

for this reason the amount of the fractions was obtained by centrifuging down, drying and weighing the coagulated proteins instead of by the Kjeldahl method. The results are shown in Table III:

TABLE III.—*Relation of the Antitryptic Power of the Serum-Albumen to the Quantity of Albumen in the Serum.*

Antitryptic index.	Albumen. Grm. in 100 c.c. of serum.
1·0	4·3
1·7	3·7
1·0	4·6
1·7	3·6

The solutions of albumen of which the inhibitory capacities were determined corresponded to definite dilutions of the sera from which they were derived, and had no relation to the quantity of albumen which they contained. This was ascertained only after the inhibitory power had been measured.

In the first pair of samples in Table III the albumen of the second serum has practically twice, and in the second pair more than twice, the inhibitory power of that of the first serum, when compared in terms of equal mass.

CONCLUSION.

In considering the bearing of these observations on the conception of the antitryptic action of the serum as a simple diversion of the enzyme from the added substrate by the proteins of the serum, we see at once that the conception in its simplest form has been invalidated by the already recognised fact that the inhibitory power is, in certain sera, practically limited to the albumen-fraction of the proteins. Obviously the diversion of the enzyme cannot be ascribed to the proteins as such. We have to recognise that there exists in this respect a difference between the more readily precipitable protein and the less readily precipitable protein, and that this difference is, in the case of human serum, a very significant one. The simplicity of the explanation is, therefore, already complicated by the fact that the inhibitory power of the albumen-fraction is found to be so much greater than the inhibitory power of the globulin-fraction. Evidently there exists some fundamental difference between the albumen and the globulin upon which this difference in their capacity for diverting trysin is dependent, and the explanation, to be adequate, must elucidate this distinction. When we pass to a consideration of the pathological variation, the conception becomes still less satisfying. For not only can we not ascribe the inhibition to a diversion of the enzyme by the protein as such, but we can now not even ascribe it to a diversion by the albumen as such. We have seen that the antitryptic power does not vary with the concentration of albumen in the serum, but that, on the contrary, it may tend to vary inversely with that concentration—a serum with an abnormally large antitryptic power may have an abnormally small

albumen content. Hence, the explanation has to cover the fact that equal masses of albumen may divert widely different quantities of trypsin from equal quantities of substrate, and has to elucidate the distinction upon which this difference is dependent. In effect, the simple explanation will have to give way to a more complex one. That the inhibition is due to a process of adsorption is probable (Hedin, 1905, 1906[1 and 2]; Young, 1918), as it is probable that most of the reactions met with in the study of immunity are due to processes of adsorption; but this adsorption is evidently dependent upon something more than the fact that the serum contains protein. It is dependent upon some at present unrecognised character of this protein—a character which may under certain conditions vary in its intensity. This character may be no more than a certain degree of dispersion of the albumen, or it may be as obscure and incomprehensible as the character upon which antitoxic action depends; but there can be no doubt that its nature, and the nature of the stimulus to its variation in disease, still constitute an important problem in pathology.

SUMMARY.

(1) The albumen-fraction of the serum-proteins is more antitryptic than the globulin-fraction, and in human serum the inhibiting factor lies almost entirely in the former.

(2) This association of the antitryptic action with the albumen-fraction applies also to the abnormal increment of antitrypsin occurring in certain conditions of disease.

(3) There is no proportional relation between the antitryptic power of the serum and the concentration, either of the total protein or of the albumen; but on the contrary, an increased inhibitory capacity may be accompanied by a diminished protein content, and especially by a diminished albumen content.

(4) The antitryptic power of the serum cannot be conceived as due to a simple diversion of the enzyme by the protein as such, but must be dependent upon some at present unrecognised character of this protein.

REFERENCES.

Bayliss, W.—(1919) 'The Nature of Enzyme Action,' London, 4th Edition, p. 148.
Von Bergmann and Meyer, K.—(1901) *Berlin. klin. Wochenschr.*, **45**, 1673.
Cathcart, E. P.—(1904) *J. Physiol.*, **31**, 497.
Cohnheim, O.—(1912) 'Enzymes,' Herter Lectures, 1910, New York, pp. 57 and 64.
Doblin, A.—(1910) *Zeitschr. f. Immunitätforsch.*, **4**, 229.
Hedin, S. G.—(1905) *J. Physiol.*, **32**, 390.—(1906)[1] *Biochem. J.*, **1**, 474.—(1906)[2] *ibid.*, **1**, 484.
Meyer, K.—(1911) *Folia Serolog.*, **7**, 471.
Teale, F. H., and Bach, E.—(1920) *Proc. R. Soc. Med.*, **13** (Sect. of Pathol.), 17.
Young, W. J.—(1918) *Biochem. J.*, **12**, 499.

THE SEROLOGICAL RELATIONSHIPS OF THE PARACHOLERA VIBRIOS TO *VIBRIO CHOLERÆ*, AND THE SEROLOGICAL RACES OF THE PARACHOLERA GROUP.

T. J. MACKIE.

From the Department of Bacteriology, University of Cape Town.

Forwarded for publication April 21st, 1922.

In 1918 two serological races of *Vibrio paracholeræ*, associated with a limited choleraic outbreak in Egypt, were described by Mackie and Storer (1918), and for purposes of future reference were designated *V. paracholeræ A* and *B*. These vibrios corresponded closely in general characters to known *V. choleræ* strains though differing from the classical cholera vibrio in being markedly hæmolytic on blood-agar plates; they were not agglutinated, even in low titres, by an antiserum to a known *V. choleræ* of epidemic origin, and agglutinating sera to both types failed to react with known *V. choleræ* strains. These paracholera strains were all highly virulent in animal experiment by intravenous and intraperitoneal injection. All the available evidence strongly supported their ætiological relationship to the choleraic disease with which they were associated. Similar organisms had been previously described and designated paracholera vibrios by Chalmers and Waterfield (1916), and by Castellani (1916).

In the original agglutination tests the serological differentiation of *V. paracholeræ A* and *B* from one another and from *V. choleræ* was well marked by the technique employed:

The emulsions were prepared in plain 0·85 per cent. saline from 18–24 hours agar-slope cultures; a series of concentrations of the antiserum were tested, ranging from relatively low dilutions up to the end-titre for the homologous organism; the mixtures of diluted serum and emulsion (0·4 c.c. each) were placed in agglutination tubes 3 in. × ¾ in., and the degree of agglutination was noted after two hours at 37° C. and also after 24 hours at room temperature. This technique had been extensively used by the writer, had always yielded uniform results, and had not exhibited apparent fallacies.

The question of serological races of *V. choleræ* has recently been studied by Douglas (1921). A number of strains from different sources were investigated and also certain non-cholera vibrios, including strains of *V. paracholeræ A* and *B*. He concluded that *V. choleræ* represented *one* serological race and embraced also the hæmolytic varieties (corresponding to the El Tor vibrio)—conclusions also arrived at by the writer in the course of certain studies of *V. choleræ* and other intestinal vibrios in Egypt.

Douglas, using formolised broth cultures as emulsions for the agglutination tests (according to Dreyer's method) and incubating at 50°–55° C. for 2 hours, found that after continued subculture on artificial medium *V. paracholeræ A* acquired the property of being agglutinated by anticholera sera, though without a corresponding result in absorption tests. Douglas also found that in the case of emulsions prepared in plain salt solution, and incubating at 50°–55° C.,

a *V. choleræ* serum agglutinated *V. paracholeræ A* and *B*, and the results with known strains of *V. choleræ* were somewhat irregular.

Such results might be interpreted as indicating a less distinct serological difference of these paracholera types from *V. choleræ* than that originally claimed for these species, and are at variance with the writer's original observations.

To quote the results of the original tests carried out by the technique referred to; none of the paracholera strains were agglutinated by a 1 : 50 dilution of an anticholera serum (Lister Institute—1916) whose end-titre was 1 : 2000; the tests were repeated after the strains had been subcultured several times, with similar results, and the strains after recovery from experimental animals still failed to react with the anticholera serum. Absorption tests, Pfeiffer's reaction and complement-deviation tests yielded corresponding results. An antiserum also to a *V. paracholeræ A* strain agglutinated homologous strains to a titre of 1 : 1600, but had no effect on a strain of *V. choleræ* or different strains of *V. paracholeræ B* in dilutions of 1 : 100. An antiserum to a strain of *V. paracholeræ B* agglutinated the homologous strains up to dilutions of 1 : 3200, but had no action on *V. choleræ* or *V. paracholeræ A* strains in titres of even 1 : 100.

The marked differences between plain and formolised emulsions as indicated by Douglas seems a factor of the greatest importance in agglutination technique, apart from their significance in regard to the particular races under consideration. The opportunity, therefore, was taken of re-testing certain paracholera strains (still kept in culture by the writer) with an antiserum to a *V. choleræ* (Egyptian origin), especially as these strains had been continuously cultivated on artificial medium for a period of four to five years.

The agglutinating serum to *V. choleræ* was prepared in the usual way and tested with the homologous strain and a *V. paracholeræ A* strain "G."

Careful comparisons were made between the results obtained when the tests were carried out by different methods. Different agglutination emulsions were compared: (1) plain saline emulsions from 18- to 24-hour agar slope cultures; (2) saline emulsions formolised—0·1 per cent. formalin; (3) formolised broth emulsions prepared by Dreyer's method. Further comparisons were also made between the results obtained after incubation at 37° C. and 55° C. for 2 hours, and readings were taken after the test mixtures had been standing at room temperature overnight following removal from the incubator.

The results are shown in Table I. Repetition of these experiments yielded similar and uniform results.

Thus, even when agglutination tests were carried out by a different technique, the effects after 2 hours were identical, *i. e.* the *V. paracholeræ A* strain was not agglutinated by the *V. choleræ* serum even in low dilutions; "coagglutination" effects were evident after 24 hours with the plain saline emulsion incubated primarily for 2 hours at 55° C., the formol-saline emulsion and the formol-broth emulsion, though absent in the case of the saline emulsion incubated first for 2 hours at 37° C. These coagglutination effects were most marked in the case of the formol-broth emulsion incubated for 2 hours at 55° C., but in degree and end-titre always fell short of the specific agglutination of the homologous type.

TABLE I.—*V. choleræ Antiserum: Serum Dilutions.*

	1:200.	1:400.	1:800.	1:1600.	1:3200.	1:6400.	1:12,800.	Control.
Saline emulsions:								
V. choleræ:								
2 hours 37° C.	++++	++++	++++	++++	++++	++	—	—
24 hours room temperature	„	„	„	„	„	+++	—	—
V. paracholeræ A:								
2 hours 37° C.	—	—	—	—	—	—	—	—
24 hours room temperature	—	—	—	—	—	—	—	—
V. choleræ:								
2 hours 55° C.	++++	++++	++++	++++	++++	++	—	—
24 hours room temperature	„	„	„	„	„	+++	++	—
V. paracholeræ A:								
2 hours 55° C.	—	—	—	—	—	—	—	—
24 hours room temperature	+++	++	—	—	—	—	—	—
Formol-saline emulsions:								
V. choleræ:								
2 hours 37° C.	++++	++++	++++	++++	++	—	—	—
24 hours room temperature	„	„	„	„	++++	++	—	—
V. paracholeræ A:								
2 hours 37° C.	—	—	—	—	—	—	—	—
24 hours room temperature	+++	++	—	—	—	—	—	—
V. choleræ:								
2 hours 55° C.	++++	++++	++++	++++	++	—	—	—
24 hours room temperature	„	„	„	„	++++	++	—	—
V. paracholeræ A:								
2 hours 55° C.	—	—	—	—	—	—	—	—
24 hours room temperature	+++	++	—	—	—	—	—	—
Formol-broth emulsions:								
V. choleræ:								
2 hours 37° C.	++++	++++	++++	++++	++	—	—	—
24 hours room temperature	„	„	„	„	+++	+++	—	—
V. paracholeræ A:								
2 hours 37° C.	—	—	—	—	—	—	—	—
24 hours room temperature	+++	+++	+	+	—	—	—	—
V. choleræ:								
2 hours 55° C.	++++	++++	++++	++++	+++	—	—	—
24 hours room temperature	„	„	„	„	„	++	—	—
V. paracholeræ A:								
2 hours 55° C.	—	—	—	—	—	—	—	—
24 hours room temperature	+++	+++	+++	++	++	—	—	—

Degree of agglutination indicated by the number of + marks; — signifies absence of agglutination.

"24 hours room temperature" refers to readings made after the tubes had remained at room temperature for 24 hours following incubation at 37° C. or 55° C.

It was thus apparent that the coagglutination effect of the *V. choleræ* serum with the *V. paracholeræ A* strain was (1) a late effect only developing slowly and therefore not comparable with the specific reaction, (2) of lesser degree and lower end-titre than the reaction with *V. choleræ*, (3) more markedly elicited by certain methods, particularly when formol-broth emulsions were used and incubated first at 55° C.

The results in no way disprove the original claim that *V. paracholeræ A* was sharply distinguished in direct agglutination test from *V. choleræ* (apart altogether from the results of absorption tests). These observations again show that the sharpest distinctions are obtained by using plain saline emulsions and incubating at 37° C.

A strain of *V. paracholeræ B* ("L") tested with the same dilutions of the anticholera serum even in the case of formol-broth emulsions and after primary incubation at 55° C. showed no coagglutination in the 24 hours' readings (Table II). This was also noted in Douglas's experiments.

Three other paracholera vibrio strains originally isolated from choleraic cases in the Egyptian Expeditionary Force, each representing different serological types (not *A* and *B*), were also tested in comparative experiment, using plain saline emulsions and formol-broth emulsions. The results are shown in Table II and correspond with those elicited with *V. paracholeræ A*.

It is also of interest to refer to and summarise here certain observations carried out by the writer in Egypt in 1916–1918 on the question of serological classification in the group of intestinal vibrios biologically allied to *V. paracholeræ A* and *B*.*

Altogether 57 strains of this group were studied corresponding in general characters to *V. paracholeræ A* and *B* as originally described; these were isolated from typical choleraic cases, from cases of non-choleraic diarrhœa, and from healthy carriers (including contacts of choleraic cases and cases convalescent from "cholera" and acute diarrhœa).

The 57 strains were all classified serologically, and among them 20 different serological types (including *A* and *B*) were identified. Eight of the serological types were represented in the series by one strain only, though the other types included multiple representatives.

The serological differentiation of these races from *V. choleræ* and from one another was well marked, as in the case of *V. paracholeræ A* and *B*. The antiserum to one type agglutinated organisms of the same type, the end-titre corresponding to that for the strain used in immunisation, but had practically no effect on strains of other types even in low titres.

It was assumed from the evidence available that vibrios of this group were responsible for a condition varying from simple diarrhœa to an acute illness clinically indistinguishable from Asiatic cholera.

The paracholera cases were for the most part sporadic or occurred in the form of limited "outbreaks."

It was impossible to establish any distinction in the types of cases associated with the different races. Only two fatal cases were noted in the series—the associated vibrios representing different serological types. Certain of the

* These investigations have not been published owing to the loss at sea in 1918 of the detailed records bearing on this work.

TABLE II.—*V. choleræ Antiserum*: *Serum Dilutions.*

	1 : 200.	1 : 400.	1 : 800.	1 : 1600.	1 : 3200.	1 : 6400.	1 : 12,800.	Control.
Saline emulsions:								
V. choleræ:								
2 hours 37° C.	++++	++++	++++	++++	+++	++	—	—
24 hours room temperature	„	„	„	„	++++	+++	—	—
V. paracholeræ B:								
2 hours 37° C.	—	—	—	—	—	—	—	—
24 hours room temperature	—	—	—	—	—	—	—	—
Vibrio "K":								
2 hours 37° C.	—	—	—	—	—	—	—	—
24 hours room temperature	—	—	—	—	—	—	—	—
Vibrio "Forrest":								
2 hours 37° C.	—	—	—	—	—	—	—	—
24 hours room temperature	—	—	—	—	—	—	—	—
Vibrio "Belah":								
2 hours 37° C.	—	—	—	—	—	—	—	—
24 hours room temperature	—	—	—	—	—	—	—	—
Formol-broth emulsions:								
V. choleræ:								
2 hours 55° C.	++++	++++	++++	++++	+++	—	—	—
24 hours room temperature	„	„	„	„	++++	++	—	—
V. paracholeræ B:								
2 hours 55° C.	—	—	—	—	—	—	—	—
24 hours room temperature	—	—	—	—	—	—	—	—
Vibrio "K":								
2 hours 55° C.	—	—	—	—	—	—	—	—
24 hours room temperature	+++	+++	+++	+++	++	—	—	—
Vibrio "Forrest".								
2 hours 55° C.	—	—	—	—	—	—	—	—
24 hours room temperature	++++	+++	+++	++	++	—	—	—
Vibrio "Belah":								
2 hours 55° C.	—	—	—	—	—	—	—	—
24 hours room temperature	+++	+++	+++	++	++	—	—	—

types including *A* and *B* were associated with severe choleraic cases, but strains of the same types were also isolated from non-choleraic diarrhœa and from healthy carriers. Some types were only associated with simple diarrhœa, and others were represented only by strains isolated from healthy carriers. These vibrio carriers were cases convalescent from "cholera" or acute diarrhœa, or men who had been in "contact" with choleraic cases though themselves presenting no history of intestinal illness.

The large number of different serological types encountered in proportion to the total number of strains examined raised the question of the actual significance of such wide serological differentiation.

In the *B. coli* group it has been noted (Mackie, 1913) that agglutinating anti-sera show a highly restricted specificity, *i. e.* for the individual strain. A closely analogous condition as regards serological reactions has also been observed in the group of hæmophilic bacteria (Maitland and Cameron, 1921), serologically identical strains being infrequent. Among the series of paracholera vibrios investigated the majority of the types recognised were represented by multiple strains. Types *A* and *B* embraced the largest number of strains in the series.

Strains of *V. paracholeræ A* and *B* were isolated in different places and at intervals of six months. Dr. Ledingham, while Consulting Bacteriologist to the Mesopotamia Expeditionary Force, informed the writer that he had examined a vibrio strain in Mesopotamia in 1917 corresponding to the *B* type (identified by agglutinating antiserum supplied to him from Egypt). Moreover the "*V. Gindha*" of Chalmers and Waterfield isolated at Port Sudan in 1915 was found to be serologically identical with two strains isolated in 1917 in the Egyptian Expeditionary Force. It is also of interest to note that Strain 10 El Tor, in the serological experiments carried out recently by Douglas, behaved like *V. paracholeræ A*.

It can hardly be said that the serological reactions in this group are analogous to that in the *B. coli* group or the group of hæmophilic bacteria; on the other hand, these organisms cannot be classified into a relatively small number of serological types as in the case of certain bacterial groups. It is apparent that no definite conclusions regarding the significance of the serological differentiation noted in this group could be arrived at without a more extensive study of these vibrios and the examination of a large number of strains, both from the standpoint of their ætiological relationships and their serological reactions. The observations summarised here, though somewhat limited, represent a further contribution to the study of serological classification among the bacteria, apart from their particular interest in connection with the group studied.

CONCLUSIONS.

(1) The paracholera vibrios comprise a group which is not serologically homogeneous, but, in addition to *V. paracholeræ A* and *B* already described, represents a considerable number of serological races precisely differentiated by agglutination reactions.

(2) By direct agglutination tests, using plain saline emulsions and incubating

at 37°C. for 2 hours, the paracholera vibrios are distinctly differentiated from *V. choleræ*.

(3) *V. choleræ* antiserum exhibits apparent co-agglutination under certain conditions towards *V. paracholeræ A* and certain similar types; this effect develops more slowly than the agglutination of the homologous organism and is of lesser degree and of lower end-titre; it is most markedly elicited when formol-broth emulsions are used and the tubes are incubated first at 55°C.

REFERENCES.

MACKIE, T. J., AND STORER, E. J.—(1918) *J. R. Army Med. Corps*, **31**, 161.
CHALMERS, A. J., AND WATERFIELD, N. E.—(1916) *J. Trop. Med. & Hyg.*, **19**, 165.
CASTELLANI, A.—(1916) *Brit. Med. J.*, **1**, 448.
DOUGLAS, S. R.—(1921) *Brit. J. Exper. Pathol.*, **2**, 49.
MACKIE, T. J.—(1913) *J. Path. & Bact.*, **18**, 137.
MAITLAND, H. B., AND CAMERON, G. C.—(1921) *Brit. J. Exper. Pathol.*, **2**, 283.

THE ANTI-BACTERICIDAL PROPERTIES OF COLLOIDAL SILICA.

PROF. S. L. CUMMINS, C.B., C.M.G., M.D.

From the Tuberculosis Department, Welsh National School of Medicine, Cardiff.

Received for publication May 25th, 1922.

DURING the course of an investigation into the anti-bactericidal action of the bile salts in connection with the culture of *B. typhosus*, the author (1911) was led to the conclusion that bile owes its power of assisting the growth of typhoid bacilli in blood cultures to its power of inhibiting the action of "complement." It appeared likely that this anti-complementary action of bile and the bile salts was attributable to their colloidal characters when in solution or perhaps when their solutions were mixed with body fluids, but this point was not definitely settled, and it was hoped that work on similar lines with known colloidal solutions might throw further light on the question. Some experiments with silica were actually contemplated in 1914, but the outbreak of war rendered them impossible. It was therefore with great interest that the author heard of the work of W. E. Gye and E. H. Kettle, of which mention was made by Dr. H. H. Dale (1921), since it appeared possible that the adjuvant action of colloidal silica in regard to the establishment of the tubercle bacillus in the tissues of experimental animals might be explicable in terms of the inhibition of bactericidal activity at the site of inoculation. On putting these suggestions to Dr. Gye early in the current year, he was so kind as to furnish the author with a supply of colloidal silica for the purpose of testing the point by experiment.

Since the inception of these investigations, my attention has been called

to a paper by Mutermilch and Bankowski (1913), who showed that both colloidal silica and also colloidal alumina have the property of adsorbing "complement," conclusions which my experiments confirm.

Further communications of the highest interest by Gye and Purdy (1922) have since appeared upon the poisonous effects of colloidal silica.

The importance thus given to this substance in relation to infection and resistance seems to justify the publication of a note on the anti-bactericidal properties of colloidal silica.

My thanks are due to Dr. W. E. Gye and Dr. H. H. Dale, F.R.S., for their kindness in providing me with a supply of colloidal silica, and to Miss C. M. Acland for her help in carrying out the experiments.

THE EFFECT OF COLLOIDAL SILICA UPON THE ANTI-BACTERIAL ACTION OF BLOOD.

Experiment 1.—5 : 4 : '22. A 24-hour broth culture of *B. typhosus* was diluted with sterile normal saline as follows:

(1) 1/100 . (2) 1/1000 . (3) 1/10,000 . (4) 1/100,000 . (5) 1/1,000,000.

Two series of Dreyer's tubes, each five in number, were prepared, containing the following reagents:

Series A:

1 vol. distilled water.
1 vol. typhoid emulsion (from dilutions 1 to 5).
2 vols. fresh blood (S.L.C.)

Series B:

1 vol. silica colloid.
1 vol. typhoid emulsion (from dilutions 1 to 5).
2 vols. fresh blood (S.L.C.).

It will be seen that, in each series, each tube from 1 to 5 contained a similar amount of fresh active blood, together with a measured volume of typhoid emulsion in which the number of bacilli diminished in tenths; the only difference being that in Series A, there was no silica colloid, but only a volume of distilled water, while, in Series B, each tube contained a volume of silica colloid.

Both series were now placed in the incubator at 37° C. for 1 hour 40 minutes. They were then withdrawn and, from each tube, a measured volume was abstracted after shaking and added to the surface of an agar slope, the agar cultures being numbered, in each series, from 1 to 5. These agar cultures were now incubated over night and the growths examined.

The results were as follows:

	Tubes (1) 1/100	(2) 1/1000	(3) 1/10,000	(4) 1/100,000	(5) 1/1 million.
Series A (without silica)	2 colonies	1 colony	sterile	sterile	sterile
Series B (with silica)	Colonies innumerable	Colonies innumerable	129 colonies	19 colonies	4 colonies

This experiment makes it clear that, while in Series A there was normal

bactericidal activity, as shown by the almost complete destruction of typhoid bacilli within the space of 1 hour 40 minutes, in Series B, the presence of the silica colloid was sufficient to preserve the bacteria from the action of the blood.

Experiment 2.—12 : 4 : '22. This experiment was a repetition of Experiment 1 except that a stronger culture was used, trypsin broth being substituted for the bouillon previously employed. Also, to make sure that there should be no fallacy introduced by retention of bacilli in clot, the whole contents of each tube, including the small clotted residue, was transferred to the surface of the agar slopes and carefully spread with a small platinum spatula. The results were as follows:

	Tubes (1) 1/100	(2) 1/1000	(3) 1/10,000	(4) 1/100,000	(5) 1/1 million.
Series A (without silica)	Many discrete colonies	About 250 colonies	About 80 colonies	10 colonies	2 colonies.
Series B (with silica)	Continuous film of growth	Continuous film	Continuous film	Innumerable discrete colonies	Many discrete colonies.

These two experiments, the second completely confirmatory of the first, make it evident that colloidal silica is very active in arresting the action of the bactericidal substances of the blood; and perhaps indicate to some extent what may take place when, as discovered by Gye, the addition of silica to a bacterial suspension enables the germs to establish themselves without check on inoculation into an experimental animal.

They suggest, too, that colloidal silica may offer a very promising medium for blood culture for organisms of the typho-colon group; and perhaps for other pathogenic organisms.

THE EFFECT OF COLLOIDAL SILICA UPON "COMPLEMENT."

It was now time to ascertain, if possible, whether this "anti-bactericidal" action of silica colloid was due, as in the case of the bile salts, to interference with the action of "complement."

Experiment 3.—A mixture of three parts silica colloid with one part of a 3·2 per cent. sodium chloride solution was prepared so as to obtain a salt content of 0·8 per cent. in the silica mixture. It may be noted in passing that this mixture, while remaining fluid during the period of the experiment, was found some days later to have assumed the consistency of a gel.

Two series of small test tubes were placed in order, each consisting of five tubes.

Reagents were added as follows:

Series A.—To each tube—

0·1 c.c. of a 5 per cent. suspension of sensitised red blood-cells.

0·1 c.c. of sterile normal salt solution.

Series B.—To each tube—

0·1 c.c. of 5 per cent. suspension of sensitised red blood-cells.

0·1 c.c. of silica colloid with 0·8 per cent. salt.

To the tubes in each series, in order from 1 to 5, was now added complement in the following amounts:
(1) 0·2 c.c. . (2) 0·3 c.c. . (3) 0·4 c.c. . (4) 0·5 c.c. . (5) 0·7 c.c.
The total volume in each tube was now raised to 1 c.c. by the addition of sterile normal saline solution.
The results were as follows:

Tubes	(1)	(2)	(3)	(4)	(5)
Series A (without silica)—					
Hæmolysis	Trace	Some	Marked	Nearly complete	Complete
Series B (with silica)—					
Hæmolysis	None	None	None	None	None

From this experiment, it was clear that the action of the "complement" had been completely inhibited by the addition of silica colloid to the mixture.
This experiment was repeated on several occasions with the same result, both with the sample of silica colloid received from Dr. Gye and a fresh sample kindly provided by Dr. Dale.
It was further ascertained that the solution was active after heating to 100° C. in steam for 30 minutes and that, while one volume of a ten-fold dilution of the colloidal silica could completely inhibit the action of two minimal hæmolytic doses of "complement," this inhibitive action was not demonstrable when the silica was diluted one hundred fold or upward.
The method of preparation of silica colloid is given by Gye and Purdy in their paper already quoted. In my experiments, the solution used was the water-clear solution, prepared by adding a dilute solution of sodium silicate to excess of strong HCl and then freeing the mixture of the HCl and NaCl by dialysis as described by these authors.

CONCLUSIONS.

1. Colloidal silica has the property of interfering with the bactericidal power of blood in marked degree.
2. It inhibits the action of "complement" in a hæmolytic mixture. This anti-complementary property is probably the character to which it owes its anti-bactericidal power.
3. These properties, together with its want of any demonstrable germicidal power, point to colloidal silica as likely to be of great use in blood cultures in cases of bacteriæmia.

REFERENCES.

Cummins, S. L.—(1911) *J. Hyg.*, **11**, 373.
Dale, H. H.—(1921) *Lancet*, **2**, 112.
Gye, W. E., and Purdy, W. J.—(1922) *Brit. J. Exper. Pathol.*, **3**, 86.
Mutermilch, S., and Bankowski, J.—(1913) *Compt. rend. Soc. de biol.*, **74**, 131.

SILICOSIS AND MINERS' PHTHISIS.

W. E. GYE AND E. H. KETTLE.*

From the National Institute for Medical Research, London, and Institute of Pathology, St. Mary's Hospital, London.

IT has long been recognised that certain occupations predispose to pulmonary tuberculosis, and that in these "dangerous" occupations the death-rate from this disease is far higher than in the general population. This is particularly so among those who work habitually in atmospheres which are laden with silica dust. The silicotic lung itself is a well-recognised pathological entity, presenting, in addition to the general increase in bulk of the connective-tissue trabeculæ common to all forms of pneumoconiosis, the development of nodules or groups of nodules of dense fibrous tissue which often surround visible particles of silica. This condition, in itself sufficiently serious, is, however, of immeasurably greater importance from the fact that the silicotic lung is far more vulnerable to invasion by bacteria, and especially by the tubercle bacillus, than the normal organ. The general facts which have been accumulated, and upon which these conclusions are based, have been summarised by Collis in the Milroy Lectures for 1915. The association of tuberculosis with silicosis in Cornwall has been established by Haldane (1904); in South Africa by Watkins Pitchford (1916) and his collaborators. Experimentally, Gardner (1920) has been able to show that a dust containing a large percentage of silica influences profoundly the course of a tuberculous infection of the lungs of guinea-pigs. Gardner used a strain of tubercle bacillus of such low virulence that "guinea-pigs infected by inhalation of this strain show lung tuberculosis, which, however, clears up with restoration of function." When guinea-pigs were dusted with a granite dust and infected with tubercle, the lungs, instead of showing "a compact, sharply circumscribed tubercle," show "a central solid mass with heavy radiating peripheral processes." There is an early and progressive fibrosis in the dusted animals which tends to retard the healing of the tuberculous lesions.

Thus, clinically, there is overwhelming evidence that silica induces a fibrosis of the lungs, which are then sensitive to tuberculosis; and experimentally there is sound evidence supporting the clinical conclusions. The explanation of these facts is yet to be sought. The greater part of the experimental work which has been done on the subject has been concerned with the mode of entry of the dust into the lungs; the literature on this is very large, and the reader is referred to Collis' lectures and to Willis (1921) for the bibliography. It may now be said that there is general agreement that the very fine particles of dust which reach the lung alveoli are taken up by phagocytes, which, according to most authors, are derived from the endothelium of the lung capillaries, and, according to others, from alveolar epithelium, and that the dust-laden phagocytes migrate to the nearest lymphoid tissue and gain access to the closed lymphatic vascular system which drains

* Working with a grant from the Medical Research Council.

the lung. Most dusts are removed from the lung in this way through the lymphatic channels, but silica, for the most part, remains *in situ*, stimulating the formation of new connective tissue.

The two problems arising out of the recorded knowledge which have occupied our attention during the last two years are:

(1) Why is silica, conspicuous and possibly alone among common dusts, capable of inducing extensive fibrosis?

(2) How does the silicotic fibrosis aid the establishment of tuberculosis?

(1) There have been many attempts made to answer the first question. For the most part, however, the answers have not been based upon experimental study. The view most often expressed is that silica owes its injurious quality to its physical properties (Watkins Pitchford, Oliver, Moore and others). Moore (1918) tabulates the properties as follows: "(1) Its heaviness; (2) its sharp vitreous fracture; (3) its great hardness and insolubility." Hardness, heaviness and sharpness of points or edges are properties which lose their significance when applied to particles of dust enclosed in the cytoplasm of wandering cells; direct mechanical injury to the lung (in the circumstances under which dust gains access) is almost inconceivable. The property of insolubility deserves more attention. Silica (SiO_2), though insoluble in all acids except hydrofluoric, is readily dissolved in alkalis. By boiling finely divided silica in a solution of Na_2CO_3 sodium silicate is formed. The statement, therefore, that silica dust is insoluble is only partially true, and the conditions under which a solution may be obtained are relevant to the problem of silicosis. Carbon particles—certainly in the test-tube—are more insoluble than silica particles, and it is universally agreed that carbon dust, unless in large amounts, is of no pathological importance. The suggestion, therefore, that an inhaled dust may, *in virtue of its insolubility*, exert a harmful action on the tissue is contrary to observed facts. Moreover, it is contrary to modern conceptions of the energetics of the body. In silicosis the injury to the lungs is a continuous process, and the amount of fibrous tissue formed is much greater than can be explained upon any mechanical process involving disturbance of microscopic anatomical relationships. Further, the fibrosis extends far beyond the near neighbourhood of the silica particles. In order that a continuous reaction between cells and a dust should ensue, molecular or submolecular contact must be established. Just as, in a physiological sense, no material is of use for energy purposes unless it is soluble, so, pathologically, with certain obvious mechanical exceptions, it is only soluble substances which can exert a substantial or a continuous effect on the tissues.

Haldane (1918) offers another answer to the question, "Why is pure quartz so difficult to remove from the lungs?" He supposes that coal-dust, shale, etc., owe their properties to a high adsorptive power, of which silica possesses little or none. Thus dust particles are attractive to dust-cells in proportion to the soluble substances adsorbed. The explanation is, therefore, that silica, adsorbing little or none of the substances which stimulate phagocytosis, is not removed from the lungs. But as we know that the way by which silica is carried into the lymphatics of the lungs is by the dust-cells this answer appears to us to be unsatisfactory.

Mellor (1918), in a written contribution to the discussion on Haldane's paper, draws attention to the surface of the small particles of silica. He states that "the surface of the fine grains of silica was sometimes found to be corroded, so that the surface appeared to be covered with a film crust of colloidal silica." It has been shown that colloidal silica is a toxic substance (Gye and Purdy, 1922), and Mellor's observation may, therefore, provide an answer to the problem which is the reverse of Haldane's, namely, that the film of colloidal silica on the particles, whilst not preventing phagocytosis, does diminish the mobility of the phagocytes, which are slowly poisoned. Mavrogordato (1922) shows that the dust-cells are immobilised in the lymphatic channels, and suggests that they become fixed and preserved by the action of the silica which they carry. Collis includes in the list of properties of silica dust, which may be significant in the solution of the problem of silicosis, the chemical property of "acidity, which, owing to the presence of the element silicon, may render the particles capable of entering into and modifying the colloidal structure of protoplasm." The meaning of this is not clear. The trend of opinion, therefore, is in favour of the view that silica causes fibrosis by acting chemically upon the tissues, and not in virtue of its physical properties.

(2) The second problem is even more difficult, and much less work has been done on it. The disorganisation of the lymphatic system in silicosis has been recognised by most authors as a potent factor in the increased susceptibility to tuberculosis. Gardner accepts this view, but adds a further explanation of the prevalence of tuberculosis among the victims of silicosis. He regards silica dust and the tubercle bacillus as irritants, and the two "acting together are capable, in a short time, of setting in motion a series of reactions on the part of the lung which neither alone is capable of initiating. By concentration of both in small foci each reinforces the action of the other, resulting in a chronic lesion which is slow to heal." Our own work on this question is the outcome of investigations into the factors influencing infection and immunity which have occupied our attention for some time. Among other substances we have used silica as a means of breaking down the normal resistance of tissues to invasion by micro-organisms, and while working with *B. tuberculosis* we obtained results which seem to have a direct bearing upon the pathology of miners' phthisis, and suggested further inquiries along these lines. The principal element in the problem of miners' phthisis is that men who are resistant to tuberculosis become susceptible with the development of silicotic fibrosis. It is important to understand the mechanism of this increased susceptibility, not merely as a part of the problem of industrial tuberculosis, but also because general tuberculosis presents a similar though more subtle problem.

Our work has been based on the known facts—(1) that colloidal silica is a cell poison; (2) that colloidal silica is the most easily formed soluble form of silica, the circulation of silica in nature depending upon this; and (3) that living matter (soil bacteria) is able to break up mineral silicates with formation of soluble silica (Bassalik, 1913). We judged it probable that the fibrosis brought about by finely-divided silica was due to the *slow* formation in the tissues of silica sol, either directly or through the intermediate formation of sodium silicate which is decomposed by carbonic acid, the sol formed acting as

a cell poison. The direct proof of this has been found impossible, and we are, therefore, compelled to fall back upon indirect evidence. The considerations which we think rule out the "physical" explanation of silicosis have already been given; the indirect evidence which supports our hypothesis is given below.

Experimental.—In approaching experimentally a subject of this complexity it is obviously necessary to proceed from the most simple experiments to those in which several factors are concerned, and we have attempted to clear the ground by studying the effects of pure silica (SiO_2), of a powder containing 40 per cent. water and of silica sol in the subcutaneous tissue. This has been followed by experiments in which tubercle bacilli have been injected in association with silica or with an inert substance (carbon). Mice were chosen as experimental animals in the earlier work, partly because of their convenience as to size, etc., but chiefly because they possess a high degree of resistance to tuberculosis, though the resistance appears to be rather different from that exhibited by man.

Our technical methods have differed from those adopted by most workers in this field. We have used subcutaneous inoculations rather than dust inhalations, because of the greater ease with which local reactions could be followed.

THE RESULTS OF INJECTING SILICA INTO THE SUBCUTANEOUS TISSUE.

In these experiments the soluble colloidal form of silica, orthosilicic acid $Si(OH)_4$ and the insoluble silica (SiO_2) were dissolved or suspended in normal neutral salt solution, and injected in doses of 2 mgrm. into the subcutaneous tissue of the flank. For the most part mice were used in these experiments, but from time to time results were compared with similar lesions produced in guinea-pigs and rabbits. The animals were killed at stated times, the local lesions carefully dissected out and fixed in Zenker's fluid or formol salt solution, and embedded in paraffin. Sometimes serial sections were prepared of the whole of the lesion, but more often it was found sufficient to examine groups of sections from different levels of the block.

It may be said at once that there is no essential difference between the reaction produced by soluble silica and that which follows the injection of the insoluble forms. The only obvious difference is that the reaction is more rapid and more transient with colloidal silica, and our findings support very strongly the view that SiO_2 becomes soluble by hydration in the tissue; but, inasmuch as with insoluble silica the reaction is somewhat delayed, the lesions produced by this substance lend themselves rather more readily to analysis than those produced by colloidal silica.

Within three hours after injection of SiO_2 a recognisable microscopic lesion is produced. It consists of an area of granular coagulation necrosis quite acellular except at the periphery, where a few of the fixed connective-tissue cells of the part can be recognised, and an occasional polynuclear leucocyte.

Five hours after the injection the lesion is more clearly defined. The centre of the focus is still quite acellular, consisting of a coagulum which has entirely replaced the fibrillar connective tissue; with different fixatives the coagulum appears granular to a greater or less degree, but these variations

are entirely mechanical, and the essential process is a coagulation necrosis of the tissue itself, and the serum poured into it. The periphery of the lesion is now more clearly defined by a zone of leucocytes, and by the crowding together of the fixed connective-tissue cells. Beyond the lesion for some distance, even in the neighbouring inguinal lymphatic gland, the capillaries are dilated and filled with blood, and present an active margination and emigration of leucocytes. It may be noted that silica injected into the tissue in this way has no specific action on the endothelium of blood-vessels. Occasionally a venule may be seen containing a delicate coagulum, but for the most part there is no thrombosis. Only in the actual lesion do the venules undergo destruction in common with muscle-fibre, nerves, and the connective tissue.

An hour or two later shows an active invasion of the periphery of the focus by leucocytes, but many of these exhibit degeneration with pyknosis and karyorrhexis of their nuclei. Further, it is obvious that the lesion has not been delimited by the reaction of the tissue, for, especially in the silica lesion, there is a spreading necrosis beyond the marginal zone of leucocytes. In the silicic acid lesion this is not always so noticeable, the explanation being that the deleterious action of the soluble poison is manifested immediately, whereas the insoluble silica becomes soluble gradually, so that its action is potent over a longer period of time.

In seventeen hours the focus is extensive. It exhibits a central acellular zone, varying somewhat in extent, but the greater part of the necrotic area is densely infiltrated with leucocytes, though these themselves are often enough degenerated and fragmented. The coagulum appears to be denser and more inspissated, presumably by the loss of fluid, and it is broken up into spherical masses which are surrounded by leucocytes. The venules are still dilated and engorged.

In forty-two hours the lesion is noticeably more localised. The general characters are the same, but there appears to be some compression of the connective tissue at the periphery, with the formation of a delicate limiting wall. Possibly also actual proliferation of fibroblasts helps in the production of this barrier.

The later stages of the lesion are characterised by the gradual shrinkage of the coagulum, the appearance at the periphery of large mononuclear cells with irregular outlines and vacuolated protoplasm, and the active proliferation of connective tissue with the formation of numerous capillaries. The final stages are those of an organising inflammatory fibrosis, but the process lasts a considerable time, and the remains of the necrotic tissue persist for ten days at least.

These are the general features of the subcutaneous silica lesion as we have observed them. It may be modified in its extent and duration, but such variations as we have observed do not appear to be of fundamental importance. While it is comparatively easy, however, to study the earlier lesions, it is much more difficult in those which have been allowed to go on to three or more days. The macroscopic lesion is itself far from easy to define, and it often happens that the microscopic preparations leave something to be desired. In any series of experiments the later results have been difficult to observe, and this has

necessitated much repetition of the work. It is possible, of course, to define the experimental area by some inert colouring matter such as carbon, but we wished to avoid complicating factors as far as possible, and have therefore relied upon composite pictures obtained from the most satisfactory stages in several experiments. There is, then, a very definite tissue reaction to silica; it is, in fact, so far as our experience of non-bacterial irritants goes, specific. The question therefore arose whether this reaction differed from others in aiding in some specific way the growth of the tubercle bacillus. A comparison between the results obtained by inoculating tubercle bacilli alone and in combination with silica gives interesting results.

INOCULATION OF TUBERCLE BACILLI ALONE.

The picture varies enormously according to whether a thick or a thin emulsion of bacilli is used.

With a thick emulsion there is a brisk reaction of the connective tissue, the vessels become dilated, and margination and emigration of leucocytes is obvious as soon as three hours after inoculation. The centre of the lesion consists of a small space in the areolar tissue containing tubercle bacilli. This is surrounded by a thin zone of leucocytes which are actively phagocytosing the bacilli, as are also the fixed connective-tissue cells of the part. Often the wandering phagocytes appear to be emigrating to the lymph-gland, but none can be distinguished within it.

In six hours after inoculation the centre of the lesion consists of an irregular space containing granular material with bacilli at the margin. Immediately around this there is a dense zone of leucocytes, many of them loaded with bacilli. Where clumps of bacilli appear, as often happens with thick emulsions, they are entirely surrounded by leucocytes. Outside this zone the connective tissue is highly cellular, the cells being leucocytes, fixed connective-tissue cells and mast-cells, and there are unusually large numbers of mast-cells around the margin of the lymph-node. Occasionally a stray phagocyte can be seen filled with bacilli, and obviously migrating towards the gland, but it is very rare to encounter such cells actually within the gland.

In nine hours there is a complete alteration in the picture. The general congestion and leucocytic infiltration has passed off, and the loose areolar tissues show little change from the normal. The lesion, however, has become definitely localised as an abscess consisting almost entirely of polynuclear leucocytes. The majority of these contain bacilli, and some are absolutely loaded with them. The general impression given is that of a much larger number of bacilli than in the earlier stages, though they are all intracellular. Around the abscess the connective-tissue fibrils are compressed to form a definite wall, though a delicate one. Outside this are leucocytes in moderate numbers—several packed with bacilli—and there appears to be slight proliferation of the fixed connective-tissue cells.

In twenty-four hours the abscess is even more clearly defined, and contains several small spherical spaces, empty except for bacilli, which are separate, not in clumps. There is intense phagocytosis. Outside the abscess there are a few isolated bacilli and cells which have phagocytosed bacilli, but there is little

general change except for some connective-tissue cell proliferation around the wall.

The later stages of the inoculation with massive doses of tubercle bacilli were studied in preparations from several different experiments, and a fairly satisfactory composite picture of the progress of the lesion has been obtained. The whole process is remarkably quiescent. There is often an indolent proliferation of the fixed connective-tissue elements at the periphery, and the abscess becomes encapsulated with a broad zone of dense fibrous tissue, which is acellular, and appears to be formed partly by compression and partly by the gradual deposit of collagen fibres. Mononuclear cells wander into the abscess in considerable numbers, ranging themselves at the periphery; they are actively phagocytic. In the course of time they replace entirely the leucocytes, which undergo gradual necrosis and lysis, sometimes to such an extent that the centre of the abscess is occupied by a structureless coagulum. In the surrounding areolar tissue occasional polynuclear and mononuclear phagocytes containing many bacilli can be seen. These eventually reach the lymph-gland and deposit their bacilli, which cause a slow, indolent reaction of the endothelial and reticular cells, which form multiple minute microscopic foci in the gland. After a period of three months or more the local lesion consists simply of a group of extremely vacuolated mononuclear cells containing granular bacilli, surrounded by dense fibrous tissue. This nodule is surrounded by a zone of fibroblasts and polyblasts of varying density.

A rather clearer picture of the reaction is obtained by the study of lesions produced by injection of extremely fine emulsions. The following is the protocol of such an experiment:

24 *hours*.—The lesion consists of a very small focus, a cavity in the areolar tissue containing in one or two sections of the series a minute coagulum, around which is a small bunch of cells. Cells are scattered sparsely in the cavity; they are rather thicker at the margin, fading off into the peripheral tissue. There are a few mononuclear elements, fixed connective-tissue cells or polyblasts, but the majority of the cells are polynuclear leucocytes. There are very few bacilli—scarcely one in each section—and they are all phagocytosed.

2 *days*.—A small diffuse focus of cell infiltration consisting of wandering cells, fibroblasts and a few polymorphs. More bacilli can be seen than in the 24-hours lesion, all phagocytosed.

3 *days*.—A very small lesion consisting chiefly of macrophages with foamy cytoplasm; a few bacilli in the cells.

7 *days* (Fig. 2).—A slight indefinite lesion consisting of an accumulation of mononuclear cells, polyblasts and fixed connective-tissue elements. Small groups of bacilli can be seen from time to time lying free in the tissues or within the bodies of fibroblasts. They consist of some ten or more bacilli tightly packed together, and suggest proliferation of the organisms *in situ*. The focus passes into a dilated lymph trunk at the side of the gland, and bacilli can be seen lying free in this with a few mononuclear cells.

The tubercle bacillus possesses only a very slight degree of pathogenicity for the mouse, and this applies equally to human and bovine strains. It can

survive and proliferate in the tissues, but it causes very little reaction. When present in small numbers the local reserves of the tissue appear sufficient to cope with it, though when injected in massive doses it calls forth a pronounced exudation of polynuclear leucocytes. It is readily phagocytosed by the fixed connective-tissue cells or polyblasts as well as by wandering leucocytes, but it can exist for a long period in the bodies of such cells without any apparent change; in fact, it seems likely that it can actually proliferate within phagocytes. It seems to cause no cell necrosis or tissue destruction, and though it may be transported, chiefly by the agency of phagocytes, to lymphatic glands and the viscera, in these secondary foci it gives rise only to a very moderate degree of tissue reaction quite unlike that seen in susceptible animals. It would seem, therefore, that tuberculosis in the mouse ought to provide a particularly favourable opportunity for studying the influence of silica in the tuberculous process.

INOCULATION OF TUBERCLE BACILLI WITH SILICA.

We have observed repeatedly that an injection of tubercle bacilli accompanied by silica into the subcutaneous tissue gives rise to a much greater local reaction than an injection of tubercle bacilli alone, and further, that general dissemination is earlier and more active. The silica effect, however, is transient, so that we have, in these experiments, inoculated fresh doses of silica weekly into the local lesion, though the dose of tubercle bacilli has not been repeated. In this way we have produced large abscesses containing enormous numbers of tubercle bacilli, far more than were originally injected, but the common silica lesion produced in this way so complicates the microscopic picture that it is impossible to arrive at a clear conception of what takes place. We have, therefore, resorted to the "early stages" method of analysis. The changes are brought out well in an experiment in which silicic acid was inoculated together with a very fine emulsion of tubercle bacilli suspended in normal saline. In twenty-four hours a considerable lesion is produced, consisting of a central fine coagulum with an outer zone of leucocytes, the whole being bounded by a thin limiting coagulum. A very few bacilli can be seen in the central zone. There is general congestion and leucocytosis in the neighbouring areolar tissue.

In two days the lesion has progressed. The central coagulum is now rather denser and contains rather more bacilli. None of these are as yet phagocytosed. The peripheral leucocytosis is as obvious as in the 1-day preparation.

In three days the central coagulum contains more bacilli and there is now some early reaction in the peripheral connective tissue.

In five days the lesion is more advanced. The central coagulum is dense and contains many more bacilli than in the earlier foci. There is much necrosis of the leucocytes in the intermediate zone, and in the external necrotic zone, which is by now extensive, there is a considerable accumulation of large mononuclear cells with an irregular outline and a vacuolated cytoplasm, probably endothelial cells. There is much new capillary formation at the periphery of the lesion and in the inner part of the external necrotic zone.

The six days' lesion (Fig. 1) is a large one, resembling in general character

the previous ones. At the periphery there is an intense fibroblastic and angioblastic reaction with a zone of condensation of collagen which suggests considerable pressure. The new granulation-tissue contains numerous wandering cells, of which macrophages with vesicular protoplasm form a considerable number. Immediately inside this reactive zone is a band of necrosis containing macrophages and degenerated leucocytes separated from the main central coagulum by a considerable zone of leucocytes, many of them in various stages of degeneration. Macrophages are especially numerous at the margin of the outer coagulated area. In these lesions the bacilli are obviously much increased in number. They can be seen in the outer necrotic zone, usually phagocytosed by macrophages, and in the intermediate leucocytic zone, but they are especially numerous in the central coagulum when they are diffusely scattered in single elements and larger closely intertwined groups.

Seven days: Here the lesion is much smaller, and, in passing, it may be pointed out that the local silica lesion shows invariably this rapid diminution in size, apparently dependent upon loss of fluid immediately after the cessation of the process of progressive tissue destruction. Essentially, however, the lesion is simply a later stages of the previous ones. There is still a central mass of necrotic coagulum, though it is obscured to some extent by leucocytes, and a peripheral necrotic zone with a limiting border of granulation-tissue. The number of mononuclear phagocytes has enormously increased and a considerable proportion of them contain bacilli. The appearances suggest that the bacilli which have been proliferating in the abscess are all engulfed by phagocytes, many of which wander out into the granulation-tissue at the margin of the lesion.

The experiments in which large doses of tubercle bacilli were used in conjunction with silica do not bring out quite so clearly the essential difference between the tubercle silica lesion and the tubercle lesion without silica, inasmuch as such a large number of bacilli is present in both series of lesions. Nevertheless, it is possible to appreciate the development of the bacilli in the necrotic tissue with the formation of large colonies.

CONCLUSIONS.

The conclusion we draw from these experiments is briefly as follows: The mouse possesses a high degree of natural immunity to tubercle, and even massive doses of bacilli injected beneath the skin can be tolerated fairly well. In course of time the organisms are encapsuled, and though a certain number of them escape this fate, being conveyed to distant parts by the action of phagocytes, the resulting lesion develops very slowly. With smaller doses of bacilli this tolerance is even more obvious. The isolated bacilli are phagocytosed by leucocytes and also by polyblasts, and in the latter elements they live practically symbiotically, proliferating freely within the cell, which shows no obvious evidence of damage from their presence. When silica is introduced along with the bacilli into the subcutaneous tissues of the mouse there is a very different result. The tissues undergo a considerable degree of necrosis, and though an intense leucocytosis is an important part of the accompanying reaction, the centre of the lesion remains acellular for several days and consists of a structureless coagulum. After a preliminary lag of two or three days

the bacilli proliferate abundantly in this coagulum, protected from the cellular defences of the body. This protection is, however, only temporary, and in a few more days the coagulum is absorbed, and the lesion becomes infiltrated with mononuclear and polynuclear phagocytes, which absorb the bacilli with avidity. In the meantime, however, a small dose of bacilli becomes a dose of considerable magnitude, and, inasmuch as an important factor in determining an infectious process is the number of organisms introduced, a simple explanation is forthcoming of the effect of silica upon such tuberculous lesions as we have described. It is too much, of course, to claim that an exactly similar process takes place in miners' phthisis, but the presence of a definite cell poison must obviously diminish the power of the tissue reaction and so favour the progressive multiplication of the infecting bacilli.

A study of subcutaneous lesions, such as we have described, suggests that the poison does not remain in particulate form at the site of inoculation for any considerable time, but is either fixed locally or is gradually removed by phagocytes and in the tissue fluids. When present it behaves as an active cell poison, but with its gradual disappearance the normal processes of repair become evident; the necrotic tissue is replaced by organising fibrous tissue, and, finally, nothing remains but a dense scar. In the silicotic lung this scarring is the prominent feature, and though tuberculosis and silicosis may co-exist in the same organ, the characteristic fibrosis is often enough the only evidence of previous exposure to silica. It is true that in the midst of the old fibrosed nodules it is possible to distinguish particles of silica, but they are so well encapsuled that it is doubtful whether they can exert any influence on the tissues, since they can scarcely pass into solution. The question therefore arises why, in the absence of tissue necrosis, or with only a minimal amount of cell destruction, the fibrotic lungs are peculiarly liable to tuberculosis? Is the scar-tissue actually less resistant and therefore more vulnerable than normal tissues, or does the successful invasion of the tubercle bacillus depend upon the disorganisation of the circulation of the tissue fluids brought about by lymphatic obstruction?

We have attempted to investigate this point experimentally, but so far our results are not conclusive, and work is still proceeding in this direction. We have found that if subcutaneous injections of small doses of silica are made into the flank of mice at weekly intervals for several weeks an extensive fibrosis is produced. If, now, tubercle bacilli are inoculated into this altered tissue, much more considerable lesions result than in normal control mice. In our experiments these results may have been due to the fact that healing was not complete—that enough necrotic or devitalised tissue was still present to serve as a nidus for the development of the bacilli. But we have found that much the same results, both in regard to fibrosis and in the subsequent reaction to tubercle bacilli, follow the repeated injections of a suspension of carbon. Carbon injected into the subcutaneous tissues of mice remains for the most part *in situ*. It is phagocytosed, but the phagocytes pass away to the neighbouring lymph-nodes only very slowly, and massive doses of carbon give rise, therefore, to blockage of lymphatic vessels and mechanical fibrosis. But though the end-results are similar, the mechanism of the production of the fibrous tissue in the two cases is, of course, entirely different. The silica

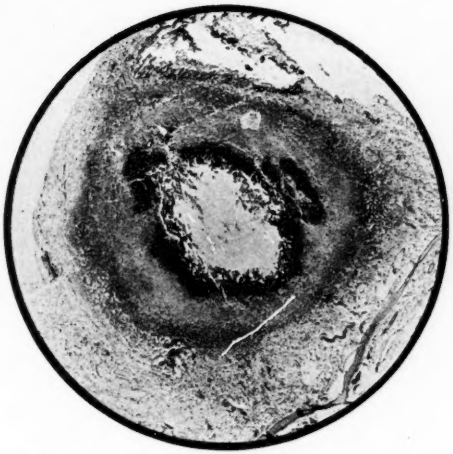


FIG. 1.—Subcutaneous lesion produced in 6 days by inoculation of 2 mgrm. of silica.

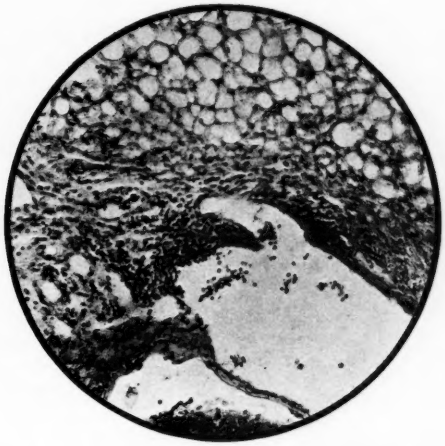


FIG. 2.—Subcutaneous lesion (7 days) following injection of fine emulsion of tubercle bacilli.

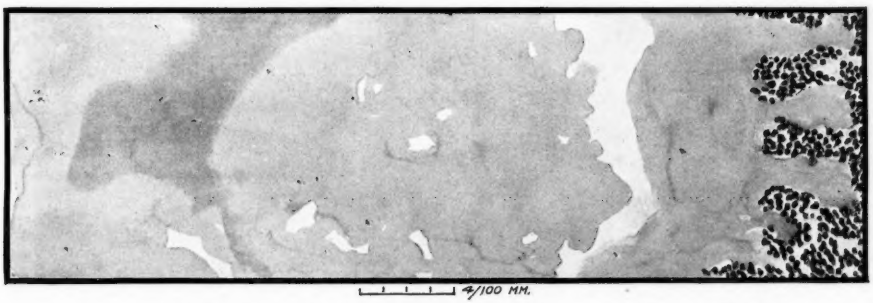


FIG. 3.—Margin of central coagulum in 3 days' silica and tubercle lesion. Early proliferation of bacilli.

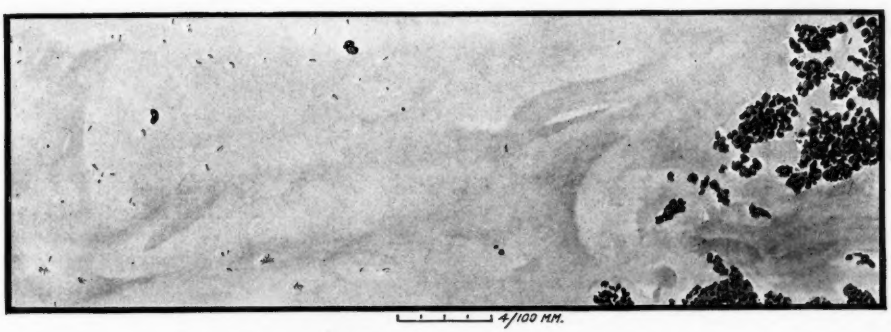


FIG. 4.—Similar lesion, but after 6 days, showing active proliferation of bacilli.

fibrosis is an end-result of a tissue destruction which cannot be produced by carbon.

It is established, of course, that coal miners are no more liable to pulmonary tuberculosis than other members of the general population, and, in point of fact, these results cannot be directly applied to the pulmonary lesions. The experimental conditions are too crude; the sudden injection of considerable localised doses of foreign substances into the tissues at irregular intervals can hardly be compared with the gradual and more or less continuous inhalation of the same substances into the lungs. Moreover, whereas the pulmonary phagocytes can deal adequately with the inert particles of carbon which reach the alveoli of the lungs, the same scavenging mechanism is not available in the subcutaneous tissues. There the carbon tends to remain within the phagocytes which have engulfed it. The phagocytes are anchored locally in considerable numbers, and must eventually die and disintegrate, and in this way sufficient pabulum may be produced to provide favourable conditions for the growth of the bacilli. Further investigations, it is hoped, may throw more light on this aspect of the problem, but at present we are unable to offer any satisfactory solution.

[The photographs from which figs. 1 and 2 have been made were taken by our colleague, Mr. J. E. Barnard, to whom we express our thanks.]

REFERENCES.

Bassalik, R.—(1913) *Ztschr. f. Gärungsphysiol.*, **3**, 15–42. Abstracted in *Bull. de l'Inst. Pasteur*, 1914, **12**, 64.

Collis, E. L.—(1915) *Publ. Health*, **28**, 292.

Gardner, L. U.—(1920) *Amer. Rev. Tubercl.*, **4**, 734–755.

Gye, W. E., and Purdy, W. J.—(1922) *Brit. J. Exp. Path.*, **3**, 75.

Haldane, J. S.—(1904) 'Report on the Health of Cornish Miners,' London (Eyre & Spottiswoode), p. 21.—(1918) *Trans. Inst. Mining Engineers*, **55**, 264–293.

Mavrogordato, A.—(1922) 'Studies in Experimental Silicosis and Other Pneumonokonioses,' *Publ. South African Inst. for Med. Res., Johannesburg.*

Mellor, J. W.—(1918) *Trans. Inst. Mining Engineers*, **55**, 264–293.

Moore, B.—(1918) *Proc. R. Soc. Med.*, **11**, 145–147.

Watkins Pitchford, W.—(1916) 'General Report; Miners' Phthisis Prevention Committee.' Pretoria.

Willis, H. S.—(1921) *Amer. Rev. Tuberc.*, **5**, 189–217.

OBSERVATIONS ON A BACTERIOLYTIC SUBSTANCE ("LYSOZYME") FOUND IN SECRETIONS AND TISSUES.

ALEXANDER FLEMING, F.R.C.S.,
Director of the Department of Systematic Bacteriology, St. Mary's Hospital,
AND
V. D. ALLISON, M.B., B.Ch.,
Research Student, St. Mary's Hospital.

From the Laboratories of the Pathological Institute, St. Mary's Hospital, London.

Received for publication 22nd July, 1922.

One of us (Fleming, 1922) has noted the occurrence in the tissues and secretions of man and animals, and in some vegetable tissues, of a bacteriolytic substance to which the name "lysozyme" has been applied. The lytic action of this substance was especially manifested on certain non-pathogenic bacteria, although it could also be observed with bacteria which were pathogenic to some of the lower animals or to man. In our experiments we have employed for the most part cultures of a large non-pathogenic coccus (which we have called *M. lysodeikticus*), as this coccus was particularly susceptible to the action of lysozyme, and furnished a convenient indicator for work in connection with its distribution and properties.

Action of lysozyme on bacteria.—This question has already been gone into at some length, and it will suffice here to say that if a lysozyme-containing material such as tears be brought into contact with a susceptible microbe such as *M. lysodeikticus*, it exercises a powerful inhibitory, bactericidal and bacteriolytic effect. One of the most remarkable features of the bacteriolytic effect is the rapidity with which it takes place. At a temperature of between 37° and 50° C. an opaque suspension of *M. lysodeikticus* in salt solution subjected to the action of a 1 per cent. solution of tears will be completely cleared in a few seconds. Again, if a small drop of tears be placed on the surface of a well-grown culture plate of this organism, in about one minute there is complete solution of the bacteria, and a clear space marks the position of the tears (Fig. 1). The microscopical appearances observed are illustrated in Fig. 2, which shows the complete disintegration of the cocci in two minutes at room temperature.

Keeping properties.—Lysozyme appears to be very stable, and maintains its activity in fluids for many weeks at room temperature. In dried material it does not appear to deteriorate, and it is to be found in very large quantities in commercial dried-egg albumen which had been prepared many months (or possibly even years) before.

Solubility.—It is soluble in water or weak saline solutions, but it is absolutely insoluble in alcohol, ether, chloroform, toluol, xylol, or acetone.

Influence of the salt content of the fluid on the lytic action.—To 1 c.c. of salt solution of various concentrations was added 0·05 c.c. of a 1 in 10 extract of nasal mucus in water and 0·05 c.c. of a thick suspension of

M. lysodeikticus. The tubes were then incubated at 45° C. and lysis noted at various times, with the results given below:

Lysis observed at times as under:

Salt concentration.	2 minutes.	10 minutes.	2 hours.	4 hours.
8 per cent.	0	0	0	0
4 ,,	0	0	0	±
2 ,,	0	±	AC	+
1 ,,	±	+	+	+
0·5 ,,	+	+	+	+
0 (control)	±	+	+	+

+ = Complete. AC = Almost complete. ± = Partial. 0 = No lysis.

It is evident therefore that high concentrations of salt inhibit the lysis, and that a concentration of 0·5 per cent. slightly favours it.

Influence of the reaction of the fluid on lysis.—To tubes containing 1 c.c. of normal salt solution *plus* acid (HCl) or alkali (NaOH) in varying amounts, was added 0·05 c.c. of a 1 in 2 saline extract of sputum and 0·05 c.c. of a suspension of *M. lysodeikticus.* The lysis noted after incubation is given below:

Concentration of alkali or acid.	Amount of lysis noted at the times indicated.							
	Alkali.				Acid.			
	5 min.	30 min.	2 hrs.	8 hrs.	5 min.	30 min.	2 hrs.	8 hrs.
N/300	0	0	0	0	0	0	0	0
N/900	0	0	0	±	0	0	0	0
N/2700	0	0	0	+	0	0	±	+
N/8100	0	±	+	+	0	±	+	+
N/24,300	0	+	+	+	+	+	+	+
Control, neutral	+	+	+	+	+	+	+	+
Control, no sputum	0	0	0	0	0	0	0	0

This shows that lysozyme is very susceptible to the reaction of the fluid, acting best in a neutral fluid and being completely or partially inhibited by very small amounts of either acid or alkali.

Effect of heat on lysozyme.—Small quantities of saliva were heated to 70° C. for varying times in a water-bath. Of this heated saliva 0·1 c.c. quantities were added to tubes containing 1 c.c. of a suspension of *M. lysodeikticus* and similar quantities of 1 c.c. of a 1 per cent. solution of starch (to make a comparison between the thermal destruction point of lysozyme and ptyalin). The tubes were then incubated for one hour and the lysis of the bacteria or the digestion of the starch noted, with the results given below:

Time during which the saliva had been heated.	Lysis of *M. lysodeikticus* observed.	Digestion of starch observed.
1 minute	±	Digested.
2 minutes	±	,,
3 ,,	±	,,
5 ,,	±	,,
7½ ,,	0	Not digested.
Unheated control	+	Digested.

This shows that in saliva lysozyme is destroyed by heat at 70° C., and that the rate of destruction is comparable with that of the diastatic ferment ptyalin.

In sputum, nasal mucus and some of the solid tissues it was found that heating for 10 minutes to 75° C. sufficed to destroy the lytic and inhibitory power.

Fig. 3 illustrates the destruction of the inhibitory power of cartilage by heat at 75° C.

In tears a higher temperature was required for complete destruction of the lysozyme. Heating to 72° C. for 10 minutes reduced the titre to one-half and 75° for the same time to one-quarter of the original, but it required boiling for over 30 minutes to completely destroy it.

Influence of temperature on the velocity of the lysis.—Lysis occurred slowly in the ice chest, so that although with 1 in 100 tears there was complete lysis of a suspension of *M. lysodeikticus*, there was no visible lysis with 1 in 1000 tears in 2 hours, whereas at 37° C. a 1 in 135,000 dilution of the same sample of tears showed complete lysis in that time. The rate of lysis is increased with the temperature up to about 60° C. After 24 hours, however, the titre is about the same, whether the temperature be that of the room, 37°, or 50° C.

Influence of certain organic substances on lysozyme.—It was found that substances such as alcohol or acetone precipitated the whole of the lysozyme from an albuminous fluid, but they did not destroy it, since if the precipitate was collected and placed in normal salt solution, it gave up to the fluid a strong bacteriolytic substance. The action of other fluids on lysozyme was investigated especially with a view to the preparation of sterile human secretions and tissues for experimental purposes.

Pus was taken from an empyema, and to quantities of about 5 c.c. an excess of toluol, thymol or chloroform was added. The tubes were tightly corked and shaken at intervals for 24 hours, when portions were removed, centrifuged, and the supernatant fluid titrated for lytic substance.

Four months later these same fluids were again titrated, having in the meantime stood on the bench in tightly corked tubes. The following results were obtained :

Preservative used.	Bacteriolytic titre after 2 hours' incubation at 45° C.									
	After 24 hours. 1 in					After 4 months. 1 in				
	100	200	400	800	1600	100	200	400	800	1600
Toluol	+	AC	...	Trace	0	+	AC	±	Trace	0
Thymol	+	+	±	,,	0	Trace	0	0	0	0
Chloroform	+	+	±	,,	0	+	AC	±	Trace	0
Control (pus without addition)	+	AC	±	,,	0	+	AC	±	,,	0

It will be seen that toluol and chloroform had no action on the lysozyme even after 4 months. Saturated thymol, on the other hand, had a deleterious effect.

The action of various fluids on the inhibitory action was investigated in

another way. Fishes' eggs (pike) were found to contain lysozyme, and it was shown that such lysozyme was essentially the same as that contained in human tissues. When these eggs were embedded in an agar plate, the surface of which was subsequently planted with *M. lysodeikticus*, there was around each egg a clear area where the growth was inhibited, and each of these areas was approximately the same size. These eggs then furnished an easy means for the investigation of the action of volatile fluids on lysozyme. It was first of all determined that a short exposure to acetone (24 hours) was without effect; then the eggs were placed in excess of acetone for purposes of dehydration. After 24 hours they were removed, dried, and small quantities were placed in various fluids and allowed to remain for 3 months, the tubes being shaken at intervals. They were then removed, thoroughly dried and imbedded in an agar plate along with some of the original eggs, which had merely had a short dehydration with acetone and which had been afterwards kept in a dry condition. The plate was planted with *M. lysodeikticus* and incubated and the result is shown in Fig. 4, from which it will be seen that the inhibitory power has not been destroyed by three months' treatment with chloroform, ether, xylol, toluol, alcohol, or acetone.

Filtration of lysozyme.—If a lysozyme-containing fluid be passed through a filter of porcelain, filter-paper, or cotton-wool, a large amount of the lytic substance is at first absorbed by the filter, so that it is only by filtering large quantities of material that any of the lysozyme passes through.

Lysozyme is also absorbed by substances such as charcoal added to the fluid.

Comparison of the lytic action on dead and living microbes.—Two identical sets of serial dilutions of tears were made. To one set was added a small quantity of a suspension of a young live culture, and to the other the same quantity of the same suspension which had been boiled for 5 minutes. The tubes were then incubated at 45° C. and it was found that lysis proceeded at the same pace in each set. There was a difference, however, in that the tubes containing the boiled microbes never became absolutely clear even after long incubation in contact with lysozyme; there always remained a faint opacity, but this opacity rapidly cleared up when a small quantity of trypsin was added, showing that it was due to a small amount of proteid matter in the bacterial suspension, which had coagulated in the process of boiling.

It appears, therefore, that the lytic action is manifested equally on living and dead organisms.

Distribution of lysozyme to M. lysodeikticus in the human body.—It has been shown (Fleming, 1922) that in all the tissues and in all the secretions and excretions tested, with the exception of cerebro-spinal fluid, sweat and urine, there exists lysozyme to *M. lysodeikticus*. To these exceptions might be added normal fæces.

It might be of interest to compare quantitatively the lytic power of body fluids and some of the tissues. The fluids were tested at various times as they became available by making serial dilutions in quantities of 1 c.c., and to each dilution adding one drop of a suspension of *M. lysodeikticus*. The comparison is not absolutely accurate in that the bacterial suspension was not always of the same strength, but the figures are sufficiently close as to give a

very fair indication of the lysozyme content. The results obtained with normal and pathological fluids are given in the following table:

Lytic Power of Normal and Pathological Fluids.

Fluid.	Highest dilution giving complete lysis.	Lowest division giving no lysis.	Time of incubation.
Cerebro-spinal fluid	—	1 in 1	Up to 24 hours.
Normal urine	—	1 in 1	,,
Sweat	—	1 in 10	,,
Blood—			
Citrated plasma	1 in 80	1 in 640	1 hour.
Serum from defibrinated blood	1 in 80	1 in 640	,,
Serum off blood-clot	1 in 40	1 in 320	,,
*Blood-serum	1 in 270	1 in 3420	,,
*Tears	1 in 40,000	1 in 325,000	,,
*Saliva	1 in 300	1 in 2700	,,
*Sputum	1 in 13,500	1 in 121,500	,,
*Nasal mucus	1 in 13,500	1 in 121,500	,,
Pleural effusion (clear)	1 in 81	1 in 2187	,,
Ascitic fluid	1 in 50	1 in 1600	,,
Hydrocele fluid	1 in 18	1 in 1438	,,
Pus (1)	1 in 700	1 in 20,000	,,
(2)	1 in 100	1 in 1600	2 hours.
Urine containing pus and albumen	1 in 32	1 in 512	1 hour.
Ovarian cyst fluid	1 in 10	1 in 160	,,
Fluid from parotid cyst	1 in 100	1 in 3200	,,
Semen	1 in 20 gave partial lysis after 3 hours.		

In connection with blood it is interesting to note that fibrin contains a considerable amount of lytic substance, and that citrated plasma and the serum from defibrinated blood both give a higher titre than does serum which has stood for any length of time on the clot. The fibrin appears to abstract from the serum a certain amount of its lytic substance, and it may well be

* From the same individual tested at the same time.

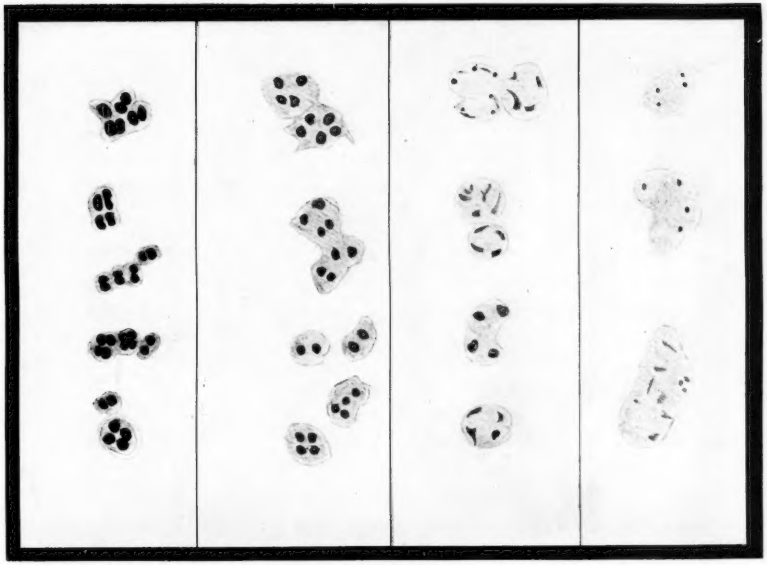
DESCRIPTION OF PLATE.

FIG. 1.—Photograph of a plate culture of *M. lysodeikticus* on which had been placed a small quantity of tears in the form of the letter *T*. Note complete clearing of that portion of the culture on which the tears had been superposed.

FIG. 2.—Microscopical appearances observed during the solution of *M. lysodeikticus* by tears. (Hiss. capsule stain.) 1. Before being acted on. 2. After 10 seconds' contact with tears 1/4 at room temperature. 3. After 30 seconds' contact. 4. After 2 minutes' contact.

FIG. 3.—Photograph of a plate showing the destruction of the inhibitory power of cartilage by 10 minutes' heating at 75° C. L. Unheated. R. Heated.

FIG. 4.—Photograph of a plate showing the inhibitory effect of pike's eggs which had been exposed to the action of various fluids for a period of 3 months.



1. 2. 3. 4.

FIG. 2.

FIG. 3.

FIG. 1.

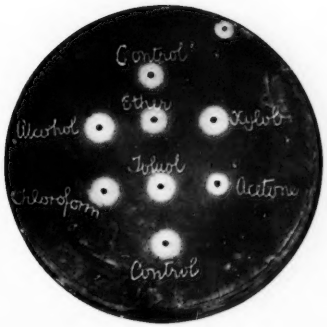


FIG. 4.

Fleming and Allison. Bacteriolytic Substance ("Lysozyme").

that this lytic power of fibrin is of importance in connection with the death of bacteria in a blood-clot.

We have investigated the question of whether the amount of lytic substance was constant in the blood-serum or tears of different individuals. Tears and blood-serum were collected from 6 laboratory workers and were titrated for lytic power with the following results:

Lysis observed after 1 hour's incubation at 45° C.

Individual.	Tears. Dilution (in thousands).							Serum. Dilution.					
	3	9	27	81	243	729	2187	10	30	90	270	810	2430
V. D. A—	+	+	AC	±	±	Trace	0	+	+	AC	±	Trace	0
A. F—	+	+	AC	±	Trace	Trace	0	+	AC	±	Trace	0	0
F. M—	+	+	±	Trace	Trace	0	0	+	+	±	Trace	Trace	0
K. N—	+	+	AC	±	Trace	Trace	0	+	AC	±	Trace	0	0
A. F. H—	+	AC	±	Trace	0	0	0	+	+	±	Trace	0	0
E. J. S—	+	AC	±	±	Trace	0	0	+	+	AC	±	Trace	0

From this it will be seen that there were definite differences between the different individuals.

It will be noticed that the titres obtained in this experiment are comparatively low. This apparently was due to the strain of coccus used being less sensitive than normal to the lysozyme. The strain used in this case had not been subcultured for two months.

The lysozyme content of certain tissues was investigated by placing the minced tissue in an excess of acetone for 24 hours for dehydration, drying the tissue, and then grinding up 1 grm. of the dry tissue with 20 c.c. of normal salt solution and allowing it to extract for 24 hours. The extract was centrifuged and the clear supernatant fluid was titrated for lytic power.* The results obtained were as follows:

Tissue.	Highest dilution giving complete lysis.	Lowest dilution showing no lysis.
Liver	1 in 400 (almost complete)	1 in 3200
Tonsil	1 in 400	1 in 6400
Kidney	1 in 100	1 in 800
Intestine	1 in 500	1 in 8000
Stomach	1 in 1000	1 in 32,000
Meninges	1 in 400 (almost complete)	1 in 3200
Skin	Less than 1 in 100	1 in 800
Cartilage	1 in 1300†	—
Turnip	1 in 4	1 in 32

It will be seen that of all the tissues tested cartilage is much the most powerful as regards lytic power. The cartilage tested in this case was derived

* This method of investigating the lytic power of tissues is almost identical with that described by Turro (1921) in his work on the bacteriolytic power of leucocyte and tissue extracts. This work was unknown to us when our experiments were carried out.

† After 5 minutes' incubation. The other results are after 1 hour's incubation.

from the articular cartilage of the patella. It may be noted that meninges possessed a considerable amount of substance, but it was found that brain tissue was very weak in this respect, although accurate titrations could not be made owing to the fact that the extracts were so opaque that bacteriolysis could not be readily observed.

In another communication (Fleming and Allison) we have shown that when bacteria are grown in the presence of a lysozyme-containing material there is at first complete inhibition of growth, but later a few scattered colonies appear, and these colonies are resistant to the lytic action of the lysozyme. Not only are they resistant to the lytic action of the tissue or secretion in contact with which they originally grew, but they are equally resistant to the lytic action of all other tissues. For instance, it was found that a strain of *M. lysodeikticus* grown in contact with nasal mucus was not more resistant to the lysozyme of nasal mucus than it was to that of liver, egg-white or turnip. It appears, then, that the lytic substance is the same in whatever tissue it occurs.

We have also shown that after solution of a large number of cocci there is an increase in the lytic content of the fluid, and that this increase in the lytic substance is dependent on the number of microbes dissolved. This is clearly shown in the following experiment:

To 2 c.c. quantities of tears (1 in 100) varying amounts of *M. lysodeikticus* were added. Incubation was carried out for 15 minutes at 45° C., when lysis was complete. The contents of the tubes were then titrated for lytic power towards *M. lysodeikticus* with the following results:

Number of cocci previously dissolved by tears.	Lysis observed after 1 hour's incubation. Dilution of tears (in thousands).						
	5	10	20	40	80	160	320
0 (control)	+	AC	±	0	0	0	0
30 million per c.c.	+	AC	±	Trace	0	0	0
300 „ „	+	+	AC	AC	±	0	0
3000 „ „	+	+	+	AC	±	0	0
30,000 „ „	+	+	+	AC	±	Trace	0

It appears, therefore, that the lysozyme acts on the bacteria in such a way that there is liberated from them a lytic substance to the same strain of bacterium.

The relation of lysozyme to "bacteriophage."—This increase in the lytic power after solution of large numbers of microbes resembles to some extent the phenomenon of the "bacteriophage," and it may be of interest here to contrast the properties of lysozyme with those of the "bacteriophage." d'Hérelle maintains that the bacteriophage is a living ultramicroscopic organism acting as a parasite on the bacteria, but such an explanation of lysozyme action would be impossible. There are variations also between the properties of the two substances which make it clear that they are essentially different. The action of the bacteriophage is relatively slow and is only manifested on young active bacterial cultures, whereas lysozyme action is very rapid and the lysis takes place with dead as well as with living bacteria. Again, the range of temperature within which the bacteriophage will act is a compara-

tively narrow one, whereas lysozyme action will take place between 4° C. and 65° C. The greatest difference, however, is that with lysozyme it has been found impossible to transmit the lytic principle in series as can be done with the bacteriophage, and that in solid cultures of bacteria *plus* lysozyme there are never seen the small round clear areas which are characteristic of the bacteriophage.

It has been mentioned above that Turro obtained in tissue extracts a bacteriolytic substance, but he does not enter sufficiently into the details of the properties of the lytic substance to say whether or not it is the same as the substance we have described under the name "lysozyme." He states, however, that the lytic principle in leucocytes was not stable, so that the extracts or even the solid substance may become inert in a few days. We have shown that lysozyme is very stable, and from the table on p. 254 it will be seen that an extract of leucocytes retains its power for at least four months.

Gengou (1921), also, has described a bacteriolytic substance which he has found in extracts of leucocytes. He found, however, that the lysin was absorbed by saturation with the microbe in contrast to the increase in the lytic substance which we have described above.

CONCLUSIONS.

There exists in human secretions and tissues (with few exceptions), as well as in animal and some vegetable tissues, a bacteriolytic substance which has properties similar to those of ferments. This lytic substance is especially active towards some non-pathogenic bacteria (and in all probability it is the cause of such bacteria being non-pathogenic), but it is active also on certain pathogenic species. So far as the indicator microbe (*M. lysodeikticus*) is concerned lysozyme is present in greatest amounts in such secretions as tears, nasal mucus, and sputum; in the tissues, especially in cartilage; while it is present in very large amount in egg-white. It may be noted that it is in just such situations that protection from bacterial contamination is required. The conjunctiva is constantly coming into contact with air-borne bacteria, as also is the nasal mucous membrane and the lining of the air-passages, and it is in the secretions covering these membranes that the lytic substance is present in greatest abundance. Cartilage also is lacking in the protection which normally comes from an abundant blood supply, while egg-white has little protection from contamination other than its inherent anti-bacterial power.

The lytic substance, lysozyme, is very stable, retaining its activity in a fluid medium at room temperature for several months. It acts equally on dead and living microbes. If small quantities are filtered the whole of the lysozyme is retained on the filter, but after the filter becomes saturated it readily passes through. Its action is manifest at temperatures between 4° C. and 65° C., but it is slower at the lower temperatures. It is not destroyed by long contact with the common organic solvents.

Strains of bacteria resistant to lysozyme are readily developed, and it is found that whatever the source of the lysozyme to which the bacteria are made resistant, they are equally resistant to the lytic action of all secretions and tissues, animal or vegetable, thus indicating the essential similarity of lysozyme from different sources. The lytic substance is not absorbed by

saturation with the indicator coccus, but, on the other hand, is increased, and the amount of the increase is dependent on the number of cocci dissolved. This increase in the lytic content is reminiscent of the "bacteriophage," but a comparison of the properties of this with lysozyme brings out some fundamental differences.

REFERENCES.

FLEMING, A.—(1922) *Proc. R. Soc.*, Ser. B, **93**, 306.
FLEMING, A., AND ALLISON, V. D.—*Ibid.* (not yet published).
GENGOU.—(1921) *Ann. de l'Inst. Pasteur*, **35**, 497.
TURRO, R.—(1921) *Compt. rend. Soc. de biol.*, **84**, 60, 290, 375, 435.

AGGLUTINATION OF WASHED RED BLOOD-CORPUSCLES BY COLLOIDAL SILICA.

PROF. S. L. CUMMINS, C.B., C.M.G., M.D.

From the Tuberculosis Department, Welsh National School of Medicine, Cardiff.

Received for publication May 25th, 1922.

DURING some preliminary experiments to ascertain the effects of colloidal silica upon phagocytosis of tubercle bacilli it was noticed that, in opsonic mixtures containing diluted blood serum, there was immediate agglutination of the red corpuscles when the washed blood suspension was added, although there seemed to be no such agglutination in preparations where the serum was more concentrated. Since the serum under examination was from the same source as the washed blood-cells, it looked, at first sight, as if the presence of the silica had brought to light some kind of paradoxical iso-agglutination of the erythrocytes, but a moment's reflection showed that the facts might be equally well explained on the assumption that the colloidal silica had some property analogous to that described for "ox colloid" by Bordet and Gay (1906). But if this were the case, it remained to be explained why there was no visible flocculation in the tubes containing the more concentrated serum. As this seemed to point rather to an interference with the agglutination by strong serum than to an agglutinating action of the serum when diluted, it was clearly necessary to ascertain whether the phenomenon could be reproduced with silica and salt solution in the absence of serum.

To settle this point, the following experiment was carried out:

Experiment I.—To a Dreyer agglutination tube, the following reagents were added:

1 vol. colloidal silica.
1 vol. of 1·5 per cent. salt solution.
2 vols. of normal salt solution (to dilute).

To this mixture was added a drop of suspension of washed blood-corpuscles. The corpuscles were at once flocculated, this effect being well brought out by blowing a portion of the mixture on to a glass slide as in blood typing. The flocculi were large and the drop of agglutinated blood presented a sharp contrast to a "control" preparation made up with the same cells and normal salt solution.

This experiment served to show that the agglutination was quite independent of the presence of serum, thus setting aside all possibility of iso-agglutination and further proving that the previous sensitisation of the red cells assumed to be necessary in the "conglutinin" phenomenon of Bordet and Gay is not needed for this silica agglutination of red corpuscles.

It is clear that the clumping is due entirely to physical properties in the mixture of cells, salt solution and colloidal silica and that the so-called "immune substances" play no part.

How, then, was the apparent inhibition of the agglutination by the higher concentrations of serum to be explained? During the first steps in the opsonic experiment to which reference has been made above, the fact had been noted that the admixture of silica colloid and human serum led, in the higher concentrations of the latter, to a marked opacity in the fluid. This opacity appeared to be accompanied by an increase in viscosity, the mixture of silica colloid and concentrated serum seeming to result in a gel. It occurred to the author that this state of gel might impede the flocculation of the red cells by mechanical obstruction and that this might be the explanation of the absence of "clumping" of the red cells with the concentrated serum. To test this assumption, the following method was tried:

Experiment II.—Three Dreyer tubes were labelled (1), (2), and (3).

To tube (1) were added:

1 vol. normal salt solution.
1 vol. serum (C.M.A.).
1 vol. of mixture of equal parts silica colloid and 1·5 per cent. salt solution.
1 vol. washed red blood-cells.

Result: No visible clumping or sedimentation of red cells.

To tube (2) were added:

2 vols. normal salt solution.
1 vol. mixed silica colloid and 1·5 per cent. salt solution.
1 vol. of washed red blood-cells.

Result: Immediate and gross clumping of the red cells.

To tube (3) were added:

1 vol. normal salt solution.
1 vol. mixed silica colloid and 1·5 per cent. salt solution.
1 vol. washed red blood-cells.

Result: Immediate and gross clumping of the red cells.

As soon as this "clumping" had become marked, 1 volume of serum (C.M.A.) was added to tube (3) and the whole thoroughly mixed by blowing bubbles of air through the fluid from a capillary pipette. This served to disperse the clumps and these now showed no tendency to re-flocculate or sediment, the appearance of tube (3) being almost identical with that of

tube (1). Examination of tubes (1) and (3) with a hand lens showed that, although no sedimentation was taking place, there was a decided tendency to minute clumping of the red cells.

From this experiment it is probable that the absence of marked clumping and agglutination in the presence of concentrated serum is due to the formation of a gell of sufficient firmness to mechanically hold the clumps from flocculating and sinking in the fluid.

To what extent does dilution of the silica colloid affect its power of clumping red blood-cells?

Experiment III.—A mixture of equal parts of silica colloid and 1·5 per cent. salt solution was prepared and from this dilutions were made in normal saline as follows: $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$ and so on up to $\frac{1}{512}$. To each tube was now added a measured volume of red blood-cells. Within five minutes at room temperature there was complete sedimentation in all the tubes up to $\frac{1}{64}$, while there was clumping, though with smaller flocculi and less sedimentation, up to $\frac{1}{512}$. A further series of dilutions to $\frac{1}{5000}$ were now prepared. In these there was no visible agglutination in 15 minutes but all showed traces of clumping after one hour.

CONCLUSIONS.

Colloidal silica solution to which sufficient salt has been added to bring the mixture up to 0·75 per cent. of NaCL brings about rapid agglutination of washed red blood-corpuscles and this result can still be obtained after considerable dilution.

The flocculation and sedimentation of washed red cells by colloidal silica as above is to a great extent prevented by the presence of concentrated human serum, this inhibition disappearing as the serum is made more dilute. The inhibition seems to depend upon the formation of a gel.

REFERENCE.

Bordet, J., and Gay, F. P.—(1906) *Ann. de l'Inst. Pasteur*, **20**, 469.